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NASAL DELIVERY OF PEPTIDES USING POWDER CARRIERS BASED ON STARCH/POLY(ACRYLIC ACID)

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Introduction and objectives

The history of insulin as a therapeutic drug dates back to 1922 when it was successfully administered in the treatment of diabetic mellitus (Brange, 1987). Until now, insulin is indispensable in the treatment of insulin-dependent diabetes mellitus (IDDM) and it is frequently indicated for non-insulin-dependent diabetes mellitus (NIDDM) as well.

Although the peroral route is considered to be the most convenient way of drug administration, it is not suitable for the delivery of peptides and proteins due to chemical or enzymatic degradation in the gastro-intestinal tract and epithelium and to the limited membrane permeability of the gastro-intestinal epithelium. In addition, peptides that have passed the gastro-intestinal barriers are exposed to a high first-pass metabolism in the liver. Furthermore, the gastro-intestinal transit time of pharmaceutical dosage forms shows a high intra- and intersubject variability. Consequently, it is difficult to develop a safe and reliable peroral delivery system for peptides having a narrow therapeutic range. This explains why most peptides are currently delivered via the parenteral route (Lee et al., 1991a; Carino and Mathiowitz, 1999).

Currently, systemic delivery of insulin is achieved by subcutaneous or intramuscular administration. Since adequate metabolic control in diabetics is essential to avoid chronic diabetes complications in IDDM patients (as shown in the Diabetes Control Complication Trial), glucose control requires an intensive insulin therapy with multiple injections a day (Jeandidier and Boivin, 1999) or by continuous infusion using an insulin pump attached to an indwelling needle inserted under the skin. However, the specific disadvantages related to the latter system limit its widespread use (Reynolds, 2000). In addition there are numerous disadvantages associated with parenteral administration: patient compliance is poor due to pain and discomfort during self-injection; absorption of subcutaneous-delivered insulin is slow and sustained and fails to mimic the physiological pulsatile pattern of endogenous-released insulin observed in non-diabetics; large intra- and interindividual variation in glycaemic control; infections may occur at the injection site (Hinchcliffe and Illum, 1999; Jeandidier and Boivin, 1999).

The disadvantages of parenteral administration have stimulated investigations of alternative routes for systemic insulin delivery such as the buccal, pulmonal, ocular, transdermal, rectal, vaginal and nasal route (Zhou and Li Wan Po, 1991; Sayani and Chien, 1996).
Among these, the nasal route has been widely investigated for peptide and protein delivery as its accessibility facilitates self-medication. The nasal cavity has a relatively large absorptive surface area due to the presence of microvilli. In addition, the endothelial basement membrane is porous and the nasal mucosa is highly vascularised ensuring rapid systemic distribution of absorbed compounds. As venous blood from the nose immediately flows into the systemic circulation the first-pass metabolism is also avoided (Cornaz and Buri, 1994; Arora et al., 2002). Furthermore, the plasma pharmacokinetic profile after nasal delivery is for some drugs similar to that of an intravenous bolus injection. For the treatment of diabetics, some studies indicated that the nasal route might allow mimicking the endogenous pulsatile secretion pattern of insulin (Hinchcliffe and Illum, 1999).

Currently, three classes of nasally administered drugs are marketed. The first class includes low molecular weight drugs for treatment of local inflammations of the nasal mucosa (sinusitis and rhinitis): e.g. naphazolin nitrate (Vasocedine®), budenoside (Rhinocort®) and beclomethasone dipropionate (Beconase Aqua®). The second class comprises low molecular weight drugs used in the treatment of migraine and intended for systemic delivery: e.g. sumatriptan (Imitrex®) and zolmatriptan (Zomig®). The third class includes a few peptide drugs like desmopressin (Minirin®), LHRH (Kryptocur®) and analogues (Suprefact® and Synarel®), and calcitonin (Miacalcic®). These peptides have sufficient nasal absorption to induce a systemic effect or have a higher stability (Verhoef et al., 1992; Behl et al., 1998).

However, nasally administered drugs (especially in the case of peptides and proteins) encounter several barriers before reaching the systemic circulation. These barriers include the mucus and epithelium (a physical barrier), the mucociliary clearance (a temporal barrier) and the enzymatic activity (a chemical barrier), resulting in a low bioavailability of peptides and proteins (Cornaz and Buri, 1994). To achieve sufficient absorption via the nasal route, simultaneous administration of peptides, absorption enhancers and/or enzyme inhibitors has been extensively investigated. Another approach to increase the nasal bioavailability has been to prolong the residence time of the formulation in the nasal cavity.

This research project will focus on powder formulations as carriers for the nasal delivery of peptides, aiming to improve the nasal bioavailability of the drug by enhancing the residence time of the drug in the nasal cavity due to the incorporation of viscosity-enhancing excipients in the formulation. Nasal powder formulations are preferred to liquid formulations as the chemical stability of the drug is increased (Lee et al., 1991b; Schipper et al., 1993). As the small volume of the nasal cavity limits the amount that can be administered, it is only suitable for the administration of low-dose or high solubility drugs via a simple liquid formulation (Ugwoke et al., 2001). In contrast, powder formulations allow to administer a larger amount of drug and excipients, increasing the residence time of the drug in the nasal cavity and possibly saturating the enzymatic degradation processes due the high drug concentrations obtained at the nasal mucosa (Illum et al., 1987). Furthermore, no preservatives are required in dry powder formulations.

The powder formulations used in this doctoral thesis are composed of a physical mixture or a spray-dried mixture of starch and poly(acrylic acid) (Carbopol®).

As a previous study (Callens, 2002) demonstrated that the spray pattern of a powder formulation from the device was influenced by the density of the formulation, the aim of the first experiment was to investigate the influence of the bulk density of the powder on the nasal bioavailability of insulin (used as model peptide) after administration to rabbits. Rabbits were selected as animal model as they are easy to handle, inexpensive and well suited for clinical trials (Gizurarson, 1990). These animals do not need to be anaesthetised and repeated blood sampling is possible due to the large total blood volume (Ugwoke et al., 2001). The blood samples can easily be collected from the ear veins (Illum, 1996) and larger amounts can be administered in comparison with smaller animal models (Gizurarson, 1993).

The influence of the addition of divalent cations to a spray-dried mixture of starch and Carbopol® on the absolute bioavailability of peptides (insulin and salmon calcitonin) in rabbits was also tested. The irritating potential of those powder formulations was evaluated using a non-invasive washing technique in rabbits and using the Slug Mucosal Irritation assay.

Stability testing of the spray-dried starch and Carbopol® stored as bulk powder was also performed.

Finally, the spray-dried mixture of starch and Carbopol® was evaluated as platform for the nasal delivery of oxymetazoline hydrochloride to healthy volunteers.

References

Arora P., Sharma S., Garg S., Permeability issues in nasal drug delivery. Drug Discov. Today 7 (2002) 967-975.

Behl C.R., Pimplaskar H.K., Sileno A.P., deMeireles J., Romeo V.D., Effects of physicochemical properties and other factors on systemic nasal drug delivery. Adv. Drug Del. Rev. 29 (1998) 89-116.

Brange J., Galenics of insulin. The physico-chemical and pharmaceutical aspects of insulin and insulin preparations. Springer-Verlag, Berlin, 1987.

Callens C., Ontwikkeling van een platform voor nasale toediening van peptiden. Doctoral thesis, Ghent University, Belgium, 2002.

Carino G.P., Mathiowitz E., Oral insulin delivery. Adv. Drug Del. Rev. 35 (1999) 249-257.

Cornaz A-L., Buri P., Nasal mucosa as an absorption barrier. Eur. J. Pharm. Biopharm. 40 (1994) 261-270.

Gizurarson S., Animal models for intranasal drug delivery. Acta Pharm. Nord. 2 (1990) 105-122.

Gizurarson S., The relevance of nasal physiology to the design of drug absorption studies. Adv. Drug Deliv. Rev. 11 (1993) 329-347.

Hinchcliffe M., Illum L., Intranasal insulin delivery and therapy. Adv. Drug Del. Rev. 35 (1999) 199-234.

Illum L., Jørgensen H., Bisgaard H., Krogsgaard O., Rossing N., Bioadhesive microspheres as a potential nasal drug delivery system. Int. J. Pharm. 39 (1987) 189-199.

Illum L., Nasal delivery. The use of animal models to predict performance in man. J. Drug. Target. 3 (1996) 427-442.

Jeandidier N., Boivin S., Current status and future prospects of parenteral insulin regiments, strategies and delivery systems for diabetes treatment. Adv. Drug Del. Rev. 35 (1999) 179-198.

Lee V.H.L., Kashi S.D., Grass G.M., Rubas W., Oral route of peptide and protein drug delivery. In: Lee V.H.L. (Ed.), Peptide and protein drug delivery, Marcel Dekker, New York, 1991a, 691-738.

Lee W.A., Narog B.A., Patapoff T.W., Wang Y.J., Intranasal bioavailability of insulin powder formulations: effect of enhancer-to-protein ratio. J. Pharm. Sci. 80 (1991b) 725-729.

Reynolds L.R., Reemergence of insulin pump therapy in the 1990s. Southern Medical Journals 93 (2000) 1157-1161.

Sayani A.P., Chien Y.W., Systemic delivery of peptides and proteins across absorptive mucosae. Crit. Rev. Ther. Drug Carrier Sys. 13 (1996) 85-184.

Schipper N.G.M., Romeijn S.G., Verhoef J.C., Merkus F.W.H.M., Nasal insulin delivery with dimethyl-β-cyclodextrin as an absorption enhancer in rabbits: powder more effective than liquid formulations. Pharm. Res. 10 (1993) 682-686.

Ugwoke M.I., Verbeke N., Kinget R., The biopharmaceutical aspects of nasal mucoadhesive drug delivery. J. Pharm. Pharmacol. 53 (2001) 3-22.

Verhoef J.C., Hermens W.A.J.J., Schipper N.G.M., Romeijn S.G., Merkus F.W.H.M., Absorption enhancement in nasal drug delivery. In: Crommelin D.J.A. and Midha K.K. (Eds.), Topics in Pharmaceutical Sciences, Medpharm, Stuttgart, 1992, 171-187.

Zhou X.H., Li Wan Po A., Peptide and protein drugs: II. Non-parenteral routes of delivery. Int. J. Pharm. 75 (1991) 117-130.

Chapter 1 The nasal musoca as absorption barrier

1.1 The nasal cavity

The inhaled air is conditioned in the nasal cavity by cleaning, warming and humidifying before reaching the fragile alveolar tissue in the lungs. The nasal cavity is divided into two approximately equal halves by the nasal septum. Each half is beginning anteriorly at the nostrils and extending posteriorly to the nasopharynx where the two halves join together. The total volume of the nasal cavity is about 15 - 20 ml and the total surface area about 150 cm^2 (Hinchcliffe and Illum, 1999). A cross-sectional view of the nasal cavity is shown in Figure 1.

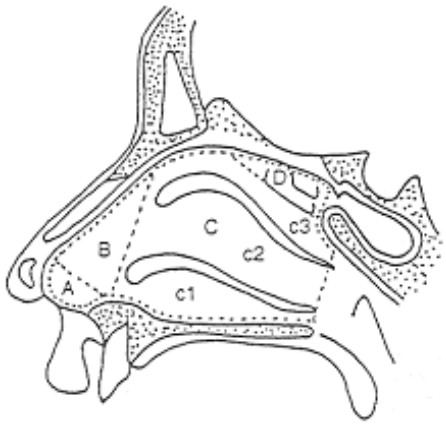


Figure 1. Anatomy of the nasal cavity (A = vestibular region, B = atrium, C = respiratory region, c1 = inferior turbinate, c2 = middle turbinate, c3 = superior turbinate, D = olfactory region). (Cornaz and Buri, 1994)

The nasal cavity can be divided into three functional regions: the vestibular (10 - 20 cm^2), the respiratory (about 130 cm^2) and the olfactory region (10 - 20 cm^2) (Hinchcliffe and Illum, 1999).

The *nasal vestibule,* situated just inside the nostrils, is covered with stratified, keratinised and squamous epithelium. The atrium forms a transepithelial region between the nasal vestibule

and the respiratory region and is anteriorly composed of stratified squamous cells and posteriorly of pseudostratified cells with microvilli (Cornaz and Buri, 1994; Mygind and Dahl, 1998; Arora et al., 2002).

The *respiratory region* occupies the majority of the nasal cavity and three turbinates (inferior, middle and superior) projecting down from the lateral wall considerably increase its surface area. The respiratory mucosa is pseudostratified having four different cell types: basal cells, ciliated and non-ciliated columnar cells and goblet cells. The basal cells, which are lying on the basement membrane, are precursor cells of the other cell types (Figure 2).

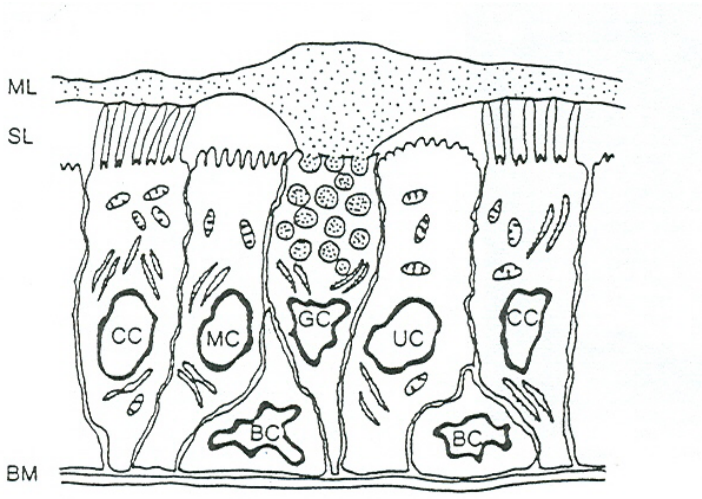


Figure 2. Nasal epithelium cells (ML = mucus layer, SL = sol layer, BM = basement membrane, CC = ciliated columnar cell, MC = non-ciliated columnar cell, BC = basal cell, GC = goblet cell, UC = undifferentiated cell). (Cornaz and Buri, 1994)

The columnar cells (ciliated as well as non-ciliated) are covered with microvilli (approximately 400 per cell), thus providing a large absorption surface to transfer nasally administered drugs from the nasal cavity into the systemic circulation. The apically located cells exhibit a high metabolic activity due to an abundance of mitochondria and have an important role in the transport of fluids.

Goblet cells, having many secretory vesicles in the cytoplasm, produce together with the submucosal glands the mucus layer on the surface of the epithelium. However, the contribution of the goblet cells to the volume of nasal secretion is probably small compared

to the submucosal glands. Their release mechanism is unclear and is probably in response to physical and chemical irritants in the microenvironments (Mygind and Dahl, 1998; Morita and Yamahara, 2004).

The *olfactory mucosa*, which is located in the roof of the nasal cavity and on the upper part of the nasal septum, contains the receptors for the sense of smell (Hinchcliffe and Illum, 1999).

The basement membrane, consisting of a layer of collagen fibrils, separates the epithelium from the lamina propria (or submucosa). The lamina propria (containing mast cells, T cells, B cells, nerves and glands) is highly vascularised and permeable, thus providing a rapid passage of fluid and dissolved substances from the tissue to the blood vessels and vice versa (Cornaz and Buri, 1994; Mygind and Dahl, 1998).

1.2 Barriers to nasal absorption

1.2.1 Mucociliary clearance

The interaction between cilia (fine contractile projections on the apical surface of most nasal epithelial cells) and mucus (a thin layer of clear nasal fluid covering the apical epithelial surface) is called the mucociliary clearance and facilitates the removal of deposited particles from the nasal cavity to the nasopharynx (Hinchcliffe and Illum, 1999). Approximately 1.5 - 2 l mucus is daily delivered by serous and seromucus glands, goblet cells, tissue fluid transudate and condensed water from expired air. Mucus is composed of water (95 - 97 %), glycoproteins (mucin) (2.5 - 3 %), electrolytes, proteins, immunoglobulins and lipids (Marttin et al., 1998). The glycoproteins give mucus its visco-elastic characteristics to provide a protective coating on the nasal epithelium and to ensure the proper functioning of the mucociliary transport. Mucus consists of two fluid layers: a viscous sol layer (periciliary fluid layer) and a viscous gel layer (mucus). The viscous sol layer containing water and soluble proteins is immediately adjacent to the epithelial surface and is covered by the viscous gel layer which is composed of water and mucins. Whereas the periciliary layer remains relatively stationairy, the cilia propel the mucus at a beat frequency of about 10 Hz towards the nasopharynx where it is removed by swallowing. The mucus turnover is about 15 - 30 min (Hinchcliffe and Illum, 1999). The efficiency of the mucociliary clearance is

determined by the length and density of the cilia and their ciliary beat frequency, by the amount of mucus and the depth of the periciliary layer and by the visco-elastic properties of the mucus (Marttin et al., 1998).

1.2.2 Permeability of the nasal epithelium

To reach their site of pharmacological activity via the blood stream, nasal administered drugs need to pass the mucus and epithelial cell layer, drug transfer across the membrane occuring via the transcellular or paracellular route.

In the transcellular route molecules permeate across the apical cell membrane, the intercellular space and the basolateral membrane by passive (diffusion, pH partition theory) or active transport (facilitated and carrier-mediated diffusion, specific transcellular transport mechanism, endocytosis). Lipophilic molecules or molecules having a specific recognition of a membrane site will preferentially be absorbed by this route. Different lipophilic drugs, such as propranolol, naloxone, buprenorphine, testosterone and 17α-ethinyloestradiol, have already shown complete absorption via the nasal route in animal models (Hinchcliffe and Illum, 1999). In case of transcellular uptake of peptides and proteins proteolytic degradation during cytoplasmatic passage can occur (Cornaz and Buri, 1994).

The paracellular route involves passive diffusion of molecules through the intercellular tight junctions and the spaces between adjacent cells. Tight junctions hold the epithelial cells together at their apical surface and are dynamic structures consisting of plasma membranes which assemble and disassemble in response to various physiological stimuli such as a decrease in extracellular calcium. As the connections between a ciliated cell and a goblet cell or between two goblet cells are relatively weak and the connections between two ciliated cells are tight, the nasal epithelium can be considered as relatively leaky. Hydrophilic, polar compounds with molecular weight up to about 1000 Dalton can cross the membrane paracellular (Cornaz and Buri, 1994). In contrast, the absorption of proteins and peptides is restricted due to their hydrophilic nature but in particular due to their high molecular weight, resulting in a low nasal bioavailability of 1 – 2 % when administered as a solution (Hinchcliffe and Illum, 1999).

1.2.3 Enzymatic degradation

The metabolic enzymes present in the nasal secretions, cytosol and lamina propria create a pseudo-first-pass effect after nasal absorption of drugs. Monooxygenases, reductases, transferases, esterases and peptidases have been identified in the nasal cavity, cytochrome P-450-dependent monooxygenase being important for the detoxification of inhaled xenobiotics. Peptides and proteins are susceptible to degradation by exo- and endopeptidases. Although Lee and Robinson (1988) reported that endopeptidases are involved in the degradation of insulin, the contribution of proteolytic activity on the low nasal bioavailability of insulin is probably minimal as insulin degradation in human nasal washing was less than 0.5 % during the period of drug absorption according to Gizurarson and Bechgaard (1991).

1.3 Strategies for improving drug absorption

Due to the low bioavailability observed after nasal delivery of peptides and proteins, several strategies have been investigated in order to improve their membrane permeability.

1.3.1 Modification of peptides

A first approach to improve nasal absorption of peptides is to modify the structure of peptides by increasing their lipophilicity in order to increase membrane permeability or to improve their stability due to a reduction of enzymatic degradation. This can be achieved by substituting specific amino acids by their equivalent D-amino acids, by derivatisation of the N-terminus or C-terminus and by cyclisation (Verhoef et al., 1992). However, this approach can affect the affinity of the molecule for the target receptors. Using this approach, the nasal bioavailability of LHRH analogues (buserelin, leuprolide and nafarelin) remained very low (2 – 3 %), but these analogues were 50 – 200 times more potent compared to native LHRH (Anik et al., 1984; Sandow and Petri, 1985).

Hashimoto et al. (1989) synthesised lipophilic palmitoyl derivatives of insulin which were less effective in reducing the glucose plasma concentrations after intramuscular injection. Asada et al. (1995) observed that chemical modification of insulin with fatty acids increased

the transport across membranes. However, the higher lipophilicity generally resulted in a lower pharmacological activity, although several derivatives had a similar pharmacological activity as native insulin.

1.3.2 Incorporation of absorption enhancers

Several studies illustrated that the incorporation of absorption enhancers like bile salts (e.g. glycocholate, taurocholate, cholate, deoxycholate, chenodeoxycholate, taurodihydrofusidate), surfactants (e.g. Laureth-9), chelating compounds (e.g. EDTA), fatty acids (e.g. sodium caprylate), phospholipids (e.g. lysophosphatidylcholine, didecanoyl-L-α-phosphatidyl-choline) and miscellanous compounds (e.g. dimethyl-β-cyclodextrin (DM-β-CD)) in a formulation enhanced the nasal absorption of insulin in animal models as well as in healthy and diabetic humans (Behl et al., 1998; Hinchcliffe and Illum, 1999; Davis and Illum, 2003). The addition of 1 % sodium taurodihydrofusidate increased the nasal bioavailability of insulin from 0.9 % to 5.2 % in rabbits and from 0.3 to 18.0 % in rats (Deurloo et al., 1989) and resulted in a bioavailability of 16.2 % in sheep (Longenecker et al., 1987). Salzman et al. (1985) described that nasal delivery of an insulin spray containing 1 % Laureth-9 lowered the plasma glucose levels to 50 % of the basal values after 45 and 120 min in healthy subjects and IDDM diabetics, respectively. Nasal delivery of insulin formulated in a liquid formulation containing DM-β-CD resulted in an absolute bioavailability of 100 % in rats, while in rabbits and humans no absorption was observed. DM-β-CD appeared to be more effective when administered in a powder formulation. An absolute nasal bioavailability of 13 %, 3.1 % and 5.1 % was found in rabbits, healthy subjects and diabetic patients, respectively (Merkus et al., 1991, 1996; Schipper et al., 1993).

The mechanism of absorption enhancement is assumed to be a combination of several effects: formation of aqueous pores in the cell membrane, disrupting the membrane integrity by their solubilising properties, increasing drug lipophilicity by ion-pairing between absorption enhancer and peptide, opening the tight junctions by chelation of extracellular divalent cations, enzyme inhibition, mucolytic activity and/or dissociation of peptides (Hinchcliffe and Illum, 1999).

Unfortunately, many absorption enhancers caused damage to nasal and other biological membranes. Laureth-9 induced a rapid and irreversible ciliostasis of human nasal cilia at a concentration of 0.3 % (Hermens et al., 1990) and 30 min after nasal administration of 1 % Laureth-9 to rats the nasal epithelium was partially disrupted showing necrosis, inflammation and exudation (Daugherty et al., 1988). Chandler et al. (1995) reported that the haemolytic activity of three lysophosphatidylcholine homologues increased with decreasing chain length of the fatty acid. EDTA, on the other hand, was well tolerated after administration to human volunteers in a concentration of 0.1 % (Batts et al., 1991). Unfortunately, its absorption promoting effect was limited as EDTA alone was not able to increase the nasal absorption of insulin (Aungst and Roger, 1988).

1.3.3 Administration of enzyme inhibitors

To protect peptides from degradation by proteases and peptidases present in the mucus, on the membrane surface and in the intercellular space enzyme inhibitors can be incorporated in the formulation. The aminopeptidase inhibitor bestatin improved the nasal absorption of desmopressin in rats, while it was ineffective in enhancing the absorption of human growth hormone and salmon calcitonin (O'Hagan et al., 1990; Morimoto et al., 1995). Another aminopeptidase inhibitor amastatin was able to increase the absorption of human growth hormone upon nasal administration to rats (O'Hagan et al., 1990). Morimoto et al. (1995) found an enhanced hypocalcemic effect in rats when the trypsin inhibitor camostat mesilate or aprotinin was administered with salmon calcitonin.
Despite the enzyme inhibiting potential of these compounds, the degree of absorption enhancement remained lower compared to other absorption enhancing strategies, as the addition of an enzyme inhibitor alone is not sufficient to obtain efficient drug absorption.

1.3.4 Formulation approach

In order to prolong the residence time of a nasal formulation in the nasal cavity (15 to 30 min due to the mucociliary clearance) and to improve the absorption efficiency a fourth approach consisted of modifying the formulation.

The simplest and most convenient way to deliver drugs nasally is via drops. However, the exact volume of dosing is difficult to determine and there is a fast drainage from the nose to the nasopharynx due to posterior deposition in the nasal cavity. Harris et al. (1986) demonstrated that reducing the droplet size using a spray device resulted in an anterior deposition and hence a slower clearance and higher extent of absorption of desmopressin.

The clearance rate of a nasal spray can be reduced by incorporation of polymers in the formulation, which increase the viscosity and/or have bio- or mucoadhesive properties (Pennington et al., 1988). A bioadhesive agent is defined as a component that interacts with biological materials through interfacial forces and is retained on them for a prolonged time period. If the biological material is a mucous membrane, the bioadhesive material is classified as a mucoadhesive (Ahuja et al., 1997). Critchley (1989) demonstrated that nasal solutions containing methyl cellulose, hydroxypropylmethyl cellulose and poly(acrylic acid) (Carbopol® 934) were able to increase the nasal bioavailability of desmopressin in rats to 27 %, 50 % and 77 %, respectively, compared to 15 % for an aqueous solution of desmopressin.

Enhanced nasal absorption can also be obtained using powder formulations instead of liquid formulations. Nagai et al. (1984) was the first to use dry powder formulations for nasal delivery of insulin. A formulation containing hydroxypropyl cellulose or neutralised Carbopol® 934 as viscosity-enhancing agent resulted in a pronounced decrease of the plasma glucose levels in rabbits after nasal administration. Callens et al. (2000, 2003) used powder blends of starch and Carbopol®, obtaining the highest nasal bioavailability (18 %) in rabbits using a spray-dried mixture of Amioca® starch/Carbopol® 974P 25/75.

Bioadhesive microsphere systems based on albumin, starch and diethylaminoethyl-dextran reduced the mucociliary clearance in human volunteers (Illum et al., 1987). Compared to a solution an improved nasal bioavailability in sheep was obtained for gentamicin (Illum et al., 1988), insulin (Farraj et al., 1990), human growth hormone (Illum et al., 1990) and desmopressin (Critchley et al., 1994) using starch microspheres. Addition of lysophosphatidylcholine to these formulations resulted in a further increase of bioavailability. Combining other absorption enhancing agents (sodium glycodeoxycholate and sodium taurodihydroxyfusidate) with starch microspheres also had a synergistic effect on the nasal

absorption of insulin, whereby the absorption of insulin and hence its biological effect was more pronounced for the powder formulation compared to a solution (Illum et al., 2001). Pereswetoff-Morath and Edman (1995) demonstrated that dextran microspheres coated with insulin were more effective than insulin-loaded microspheres. For the latter, water had to penetrate into the core of the microsphere to obtain complete swelling of the dextran matrix prior to dissolution and diffusion of insulin out of the sphere. Furthermore, poly(acrylic acid) and hyaluronic acid microspheres have also been investigated for the nasal delivery of peptides (Vidgren et al., 1992; Illum et al., 1994a).

Several mechanisms of absorption enhancement have been proposed for powder formulations containing viscosity-enhancing and/or bioadhesive agents. They absorb water and swell to form a gel after contact with the nasal mucosa, thus increasing the residence time of the drug in the nasal cavity. The water absorption from the nasal mucosa could also shrink the epithelial cells, resulting in a transient opening of the tight junctions (Pereswetoff-Morath, 1998). Some authors suggested that the calcium-binding capacity of polymers such as starch microspheres, dextran microspheres and Carbopol® may be involved in the drug absorption enhancement as in-vitro studies revealed that depletion of extracellular calcium resulted in the opening of the tight junctions (Junginger, 1990; Oechslein et al., 1996).

Chitosan, derived from chitin by partial deacetylation, is a linear cationic polysaccharide consisting of copolymers of β-1,4-linked monomers of glucosamine and N-acetyl glucosamine and is able to enhance the nasal delivery of insulin. Illum (1999) reported that liquid formulations containing 0.5 % chitosan resulted in a rapid and reproducible increase of serum insulin after nasal application in human volunteers, obtaining an absolute bioavailability of 7 %. Studies in sheep indicated that the bioavailability might be improved by administering a chitosan powder formulation instead of a liquid formulation. Human growth hormone formulated in chitosan powder or granules resulted in a nasal bioavailability of 14 % and 15 %, respectively, relative to a subcutaneous injection (Cheng et al., 2005). Nasal administration of nanoparticles of chitosan induced significantly lower plasma glucose levels in rabbits in comparison with a liquid chitosan formulation (Fernandez-Urrusuno et al., 1999). However, following the study of Dyer et al. (2002) similar nanoparticles were not effective in decreasing the plasma glucose levels what was contributed to the lower dose of insulin in the nanoparticles. In-vitro measurements revealed that the bioadhesive properties of

chitosan-ethylcellulose microparticles were higher compared to pure chitosan (Martinac et al., 2005). Also microparticles consisting of chitosan and sodium alginate exhibited good mucoadhesive properties with an improved drug permeation ex-vivo compared to microparticles without sodium alginate (Gavini et al., 2005). The absorption enhancing potential of chitosan is contributed to interactions between the positive charges of the polymer and the negatively charged sialic acid residues in the mucus layer (Illum et al., 1994b). This results in a reduced mucociliary clearance and in structural changes of the tight junctions with a transient opening of the paracellular pathway (Artursson et al., 1994).

Besides viscosity-enhancing or bioadhesive polymers water-insoluble powders can also be used as carrier for the nasal delivery of peptides. Ishikawa et al. (2001, 2002) increased the nasal bioavailability of ($Asu^{1,7}$)-eel calcitonin using water-insoluble calcium carbonate ($CaCO_3$) as a carrier. Haruta et al. (2003) demonstrated that porous $CaCO_3$ spheres were even more effective in enhancing the nasal bioavailability of insulin than conventional $CaCO_3$ powder. Teshima et al. (2002) increased the maximum plasma concentrations of glucagon in healthy volunteers from 111 ± 59.3 to 358 ± 68.0 pg/ml following nasal administration of powder formulations without and with microcrystalline cellulose as carrier, respectively. Suzuki and Makino (1999) observed the highest absorption of calcitonin and leuprolide in rabbits when a powder mixture of microcrystalline cellulose and hydroxypropyl cellulose was used. It has been shown that insoluble powders exhibit a longer residence time in the nasal cavity compared to liquid formulations, which may result in an enhanced absorption.
On the other hand, powder formulations containing water-soluble compounds such as lactose did not succeed in enhancing the nasal absorption of peptides (Nagai et al., 1984; El-Shafy et al., 2000).

1.4 References

Ahuja A., Khar R.K., Ali J., Mucoadhesive drug delivery systems. Drug Dev. Ind. Pharm. 23 (1997) 489-515.

Anik S.T., McRae G., Nerenberg C., Worden A., Foreman J., Hwang J.Y., Kushinsky S., Jones R.E., Vickery B., Nasal absorption of nafarelin acetate, the decapeptide [D-Na1(2)^{6}]LHRH, in rhesus monkeys I. J. Pharm. Sci. 73 (1984) 684-685.

Arora P., Sharma S., Garg S., Permeability issues in nasal drug delivery. Drug Discov. Today 7 (2002) 967-975.

Artursson P., Lindmark T., Davis S.S., Illum L., Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm. Res. 11 (1994) 1358-1361.

Asada H., Douen T., Waki M., Absorption characteristics of chemically modified insulin derivatives with various fatty acids in the small and large intestine. J. Pharm. Sci. 84 (1995) 682-687.

Aungst B.J., Roger N.J., Site dependence of absorption promoting actions of laureth-9, sodium salicylate, disodium EDTA and aprotinin on rectal, nasal and buccal insulin delivery. Pharm. Res. 5 (1988) 305-308.

Batts A.H., Mariott C., Martin G.P., Bond S.W., Greaves J.L., Wilson C.G., The use of radiolabelled saccharin solution to monitor the effect of the preservatives thiomersal, benzalkonium chloride and EDTA on the human clearance. J. Pharm. Pharmacol. 43 (1991) 180-185.

Behl C.R., Pimplaskar H.K., Sileno A.P., Xia W.J., Gries W.J., deMeireles J.C., Romeo V.D., Optimization of systemic nasal drug delivery with pharmaceutical excipients. Adv. Drug Del. Rev. 29 (1998) 117-133.

Callens C., Remon J.P., Evaluation of starch–maltodextrin–Carbopol® 974 P mixtures for the nasal delivery of insulin in rabbits. J. Contr. Rel. 66 (2000) 215-220.

Callens C., Pringels E., Remon J.P., Influence of multiple nasal administrations of bioadhesive powders on the insulin bioavailability, Int. J. Pharm. 250 (2003) 415-422.

Chandler S.G., Thomas N.W., Illum L., Nasal absorption in the rat. IV. Membrane activity of absorption enhancers. Int. J. Pharm. 117 (1995) 139-146.

Cheng Y.H., Dyer A.M., Jabbal-Gill I., Hinchcliffe M., Nankervis R., Smith A., Watts P., Intranasal delivery of recombinant human growth hormone (somatropin) in sheep using chitosan-based powder formulations. Eur. J. Pharm. Sci. 26 (2005) 9-15.

Cornaz A-L., Buri P., Nasal mucosa as an absorption barrier. Eur. J. Pharm. Biopharm. 40 (1994) 261-270.

Critchley H., Intranasal drug delivery. Doctoral thesis, University of Nottingham, Nottingham, UK, 1989.

Critchley H., Davis S., Farraj N., Illum L., Nasal absorption of desmopressin in rats and sheep. Effect of a bioadhesive microsphere delivery system. J. Pharm. Pharmacol. 46 (1994) 651-656.

Davis S.S., Illum L., Absorption enhancers for nasal drug delivery. Clin. pharmacokinetics 45 (2003) 1107-1128.

Daugherty A.L., Liggitt H.D., McCabe J.G., Moore J.A., Patton J.S., Absorption of recombinant methionyl-human growth hormone (Met-hGH) from rat nasal mucosa. Int. J. Pharm. 45 (1988) 197-206.

Deurloo M.J.M., Hermens A.J.J., Romeyn S.G., Verhoef J.C., Merkus F.W.H.M., Absorption enhancement of intranasally administered insulin by sodium taurodihydrofusidate (STDHF) in rabbits and rats. Pharm. Res. 6 (1989) 853-856.

Dyer A.M., Hinchcliffe M., Watts P., Castile J., Jabbal-Gill I., Nankervis R., Smith A., Illum L., Nasal delivery of insulin using novel chitosan based formulations: a

comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. Pharm. Res. 19 (2002) 998-1008.

El-Shafy M.A., Kellaway I.W., Taylor G., Dickinson P.A., Improved nasal bioavailability of FITC-Dextran (Mw 4300) from mucoadhesive microspheres in rabbits. J. Drug Target. 7 (2000) 355-361.

Farraj N., Johansen B., Davis S., Illum L., Nasal administration of insulin using bioadhesive microspheres and lysophosphatidylcholine as a delivery system. J. Contr. Rel. 13 (1990) 253-261.

Fernandez-Urrusuno R., Calvo P., Remunan-Lopez C., Vila-Jato J.L., Alonso M.J., Enhancement of nasal absorption of insulin using chitosan nanoparticles. Pharm. Res. 16 (1999) 1576-1581.

Gavini E., Rassu G., Sanna V., Cossu M., Giunchedi P., Mucoadhesive microspheres for nasal administration of an antiemetic drug, metoclopramide: in-vitro/ex-vivo studies. J. Pharm. Pharmacol. 57 (2005) 287-294.

Gizurarson S., Bechgaard E., Study of nasal enzyme activity towards insulin in vitro, Chem. Pharm. Bull. 39 (1991) 2155-2157.

Harris A.S., Nilsson I.M., Wagner Z.G., Alkner U., Intranasal administration of peptides: nasal deposition, biological response and absorption of desmopressin. J. Pharm. Sci. 75 (1986) 1085-1088.

Haruta S., Hanafusa T., Fukase H., Miyajima H., Oki T., An effective absorption behaviour of insulin for diabetic treatment following intranasal delivery using porous spherical calcium carbonate in monkeys and healthy human volunteers. Diabetes Technol. Ther. 5 (2003) 1-9.

Hashimoto M., Takada K., Kiso Y., Muranishi S., Synthesis of palmitoyl derivatives of insulin and their biological activities. Pharm. Res. 6 (1989) 171-176.

Hermens W.A.J.J., Hooymans P.M., Verhoef J.C., Merkus F.W.H.M., Effect of absorption enhancers on human nasal tissue ciliary movement in vitro. Pharm. Res. 7 (1990) 144-146.

Hinchcliffe M., Illum L., Intranasal insulin delivery and therapy. Avd. Drug Del. Rev. 35 (1999) 199-234.

Illum L., Jorgensen H., Bisgaard H., Bioadhesive microspheres as a potential nasal drug delivery system. Int. J. Pharm. 39 (1987) 189-199.

Illum L., Farraj N., Critchley H., Davis S., Nasal administration of gentamicin using a novel microsphere delivery system. Int. J. Pharm. 45 (1988) 261-265.

Illum L., Farraj N., Davis S., Johansen B., O'Hagan D., Investigation of the nasal absorption of biosynthetic human growth hormone in sheep - use of a bioadhesive microsphere delivery system. Int. J. Pharm. 63 (1990) 207-211.

Illum L., Farraj N., Fisher A., Gill I., Miglietta M., Benedetti L., Hyaluronic acid ester microspheres as a nasal drug delivery system for insulin. J. Contr. Rel. 29 (1994a) 133-141.

Illum L., Farraj N., Davis S.S., Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. 11 (1994b) 1186-1189.

Illum L., Bioadhesive formulations for nasal peptides delivery. Drugs Pharm. Sci., 98 (1999) 507-537.

Illum L., Fisher A.N., Jabbal-Gill I., Davis S.S., Bioadhesive starch microspheres and absorption enhancing agents act synergistically to enhance the nasal absorption of polypeptides. Int. J. Pharm. 222 (2001) 109-119.

Ishikawa F., Katsura M., Tamai I., Tsuji A., Improved nasal bioavailability of elcatonin by insoluble powder formulation. Int .J. Pharm. 224 (2001) 105-114.

Ishikawa F., Murano M., Hiraishi M., Yamaguchi T., Tamai I., Tsuji A., Insoluble powder formulation as an effective nasal drug delivery system. Pharm. Res. 19 (2002) 1097-1104.

Junginger H.E., Bioadhesive polymer systems for peptide delivery. Acta Pharm. Technol. 36 (1990) 110-126.

Lee V.H.L., Robinson J.R., Enzymatic barriers to peptide and protein absorption. CRC Crit. Rev. in Ther. Drug Carrier Syst. 5 (1988) 69-97.

Longenecker J.P., Moses A.C., Flier J.S., Silver R.D., Carey M.C., Dubovi E.J., Effects of sodium taurodihydrofusidate on nasal absorption of insulin in sheep. J. Pharm. Sci. 76 (1987) 351-355.

Martinac A., Filipovic-Grcic J., Voinovich D., Perissutti B., Franceschinis E., Development and bioadhesive properties of chitosan-ethylcellulose microspheres for nasal delivery. Int. J. Pharm. 291 (2005) 69-77.

Marttin E., Schipper N.G.M., Verhoef J.C., Merkus F.W.H.M., Nasal mucociliary cleareance as a factor in nasal drug delivery. Adv. Drug Del. Rev. 29 (1998) 13-38.

Merkus F.W.H.M., Verhoef J.C., Romeijn S.G., Schipper N.G.M., Absorption enhancing effect of cyclodextrins on intranasally administered insulin in rats. Pharm. Res. 8 (1991) 588-592.

Merkus F.W.H.M., Schipper N.G.M., Verhoef J.C., The influence of absorption in normal and diabetic subjects. J. Contr. Rel. 41 (1996) 69-75.

Morimoto K., Miyazaki M., Yamaguchi H., Kakemi M., Effects of proteolytic enzyme inhibitors on nasal absorption of salmon calcitonin in rats. Int. J. Pharm. 113 (1995) 1-8.

Morita T., Yamahara H., Peptides and proteins – Nasal absorption. In: Swarbrick J., Boylan J.C. (Eds.), Encyclopedia of pharmaceutical technology, Marcel Dekker Inc., New York, 2004, 1-14.

Mygind N., Dahl R., Anatomy, physiology and function of the nasal cavities in health and disease. Adv. Drug Del. Rev. 29 (1998) 3-12.

Nagai T., Nishimoto Y., Nambu N., Suzuki Y., Sekine K., Powder dosage form of insulin for nasal administration. J.Contr. Rel. 1 (1984) 15-22.

Oechslein C.R., Fricker G., Kissel T., Nasal delivery of octreotide: absorption enhancement by particulate carrier systems. Int. J. Pharm. 139 (1996) 25-32.

O'Hagan D.T., Critchley H., Faraj N.F., Fisher A.N., Johansen B.R., Davis S.S., Illum L., Nasal absorption enhancers for biosynthetic human growth hormone in rats. Pharm. Res. 7 (1990) 772-776.

Pennington A.K., Ratcliffe J.H., Wilson C.G., Hardy J.G., The influence of solution viscosity on nasal spray depostion and clearance. Int. J. Pharm. 43 (1988) 221-224.

Pereswetoff-Morath L., Edman P., Dextran microspheres as a potential nasal drug delivery system for insulin – in vitro and in vivo properties, Int. J. Pharm. 124 (1995) 37-44.

Pereswetoff-Morath L., Microspheres as nasal drug delivery system. Adv. Drug Del. Rev. 29 (1998) 185-194.

Salzman R., Manson J.E., Griffing G.T., Intranasal aerosolized insulin. Mixed-meal studies and long-term use in type I diabetes. New Engl. J. Med. 312 (1985) 1078-1084.

Sandow J., Petri W., Intranasal administration of peptides: Biological activity and therapeutic efficacy. In: Chien Y.W., (Ed.), Transnasal Systemic Medications, Elsevier, Amsterdam, 1985, 183-199.

Schipper N.G.M., Romeijn S.G., Verhoef J.C., Merkus F.W.H.M., Nasal insulin delivery with dimethyl-β-cyclodextrin as an absorption enhancer in rabbits: powder more effective than liquid formulations. Pharm. Res. 10 (1993) 682-686.

Suzuki Y., Makino Y., Mucosal drug delivery using cellulose derivatives as a functional polymer. J. Contr. Rel. 62 (1999) 101-107.

Teshima D., Yamauchi A., Makino K., Kataoka Y., Arita Y., Nawata H., Oishi R., Nasal glucagons delivery using microcrystalline cellulose in healthy volunteers. Int. J. Pharm. 33 (2002) 61-66.

Verhoef J.C., Hermens W.A.J.J., Schipper N.G.M., Romeijn S.G., Merkus F.W.H.M., Absorption enhancement in nasal drug delivery. In: Crommelin D.J.A., Midha K.K. (Eds.), Topics in pharmaceutical sciences, Medpharm, Stuttgart, 1992, 171-187.

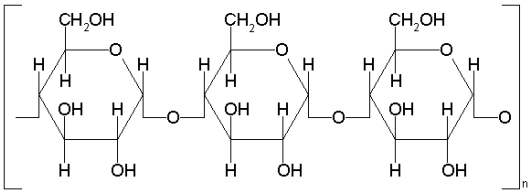
Vidgren P., Vidgren M., Arppe J., Hakuli T., Laine E., Paronen P., In vitro evaluation of spray-dried mucoadhesive microspheres for nasal administration, Drug Dev. Ind. Pharm. 18 (1992) 581-597.

Chapter 2 Composition of powder formulations

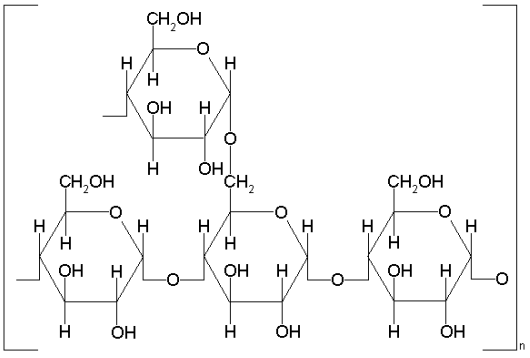
2.1 Starch/Carbopol® 974P mixtures

2.1.1 Drum Dried Waxy Maize starch/Carbopol® 974P mixtures

Starch is a polysaccharide composed of α-D-glucopyranosyl units. Two types of D-glucopyranose polymers can be distinguished: amylose and amylopectin (Figure 1).



AMYLOSE



AMYLOPECTIN

Figure 1. Chemical structure of starch components.

Amylose is a linear polymer wherein the glucopyranosyl units are linked by α-D-(1,4) glucosidic bonds. These molecules can consist of 100 - 2000 glucose units (Newman et al., 2002). Aqueous solutions of amylose are very unstable due to intermolecular attractions and associations of neighbouring amylose molecules. This leads to viscosity

increase, retrogradation and, under specific conditions, precipitation of amylose particles (Young, 1984).

Amylopectin has a branched structure, each branch being composed of 12 to 25 glucopyranosyl units connected with α-D-(1,4) linkages, the different branches linked by α-D-(1,6) glucosidic bonds (Newman et al., 2002). Aqueous solutions of amylopectin are characterised by a high viscosity, clarity, stability and resistance to gelling (Young, 1984).

Depending on their origin the amylose/amylopectin ratio of the starches will vary, most naturally occurring starches containing ± 30 % amylose. 'Waxy' maize starch is a genetically modified starch containing ± 100 % amylopectin (Whistler and Daniel, 1984).

Drum Dried Waxy Maize starch (DDWM) (Eridiana-Béghin Say Cerestar, Vilvoorde, Belgium) is a thermally modified starch formed by simultaneous cooking and drying of native maize starch on heated rolls ('drums') in order to obtain a pregelatinised starch (Wade and Weller, 1994).

Carbopol® 974P (C 974P) (Noveon, Cleveland, Ohio, USA) is a synthetic, water swellable, high molecular weight, cross-linked acrylic acid-based polymer (Figure 2). Carbopol® 974P is cross-linked with allyl pentaerythritol and is polymerised in ethyl acetate (Ahuja et al., 1997). After dispersion in water, Carbopol® partially swells, increasing the viscosity of the dispersion. The viscosity is pH dependent: the lowest viscosity is obtained in acid media and a maximum is reached at pH 7.4. Above their pKa-value (6.0 ± 0.5) the carboxyl groups become ionised and electrostatic repulsion between the negative charges causes uncoiling and expansion of the molecule, resulting in swelling of the polymer and gel formation.
Carbopol® 974P is a pharmaceutical grade polymer used as controlled release agent in tablets, as bioadhesive, as thickener, as suspending agent and emulsion stabiliser (Singla et al., 2000).

Nasal powder formulations based on a physical mixture of Drum Dried Waxy Maize starch/ Carbopol® 974P (DDWM/C 974P) at a ratio 95/5 were used.

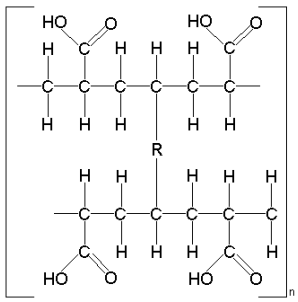


Figure 2. Structure of Carbopol® 974P (R = allyl pentaerythritol)

2.1.2 Spray-dried Amioca® starch/Carbopol® 974P mixtures

Amioca® starch (National Starch and Chemical Company, Bridgewater, New Jersey, USA) is a maize starch mainly consisting of amylopectin (waxy maize). Amioca® starch is frequently used as native thickener and texturising agent in the food industry.

Prior to spray-drying, the Amioca® starch was pregelatinised by jet cooking in a custom-made jet cooker. The temperature was set at 138 °C, the pressure at 3.1 – 3.2 bar and the flow rate at 1.2 – 1.5 l/min. Steam jet cooking is an effective manner to rapidly form an aqueous starch solution: a slurry of granular starch is brought in contact with high pressure steam, leading to gelatinisation, disruption and solubilisation of the granules (Byars, 2003). Phase contrast optical microscopy (Olympus BH2-UMA, Olympus, New York, USA) was used to verify that pregelatinisation was complete and no granules remained.
To produce the spray-dried mixtures, an aqueous mixture of the jet cooked/pregelatinised Amioca® starch and Carbopol® 974P was spray-dried in a Bowen spray-dryer model BE-1393 (Arnold Equipment Company, Cleveland, Ohio, USA). Mixtures were spray-dried at a ratio 25/75 and 85/15 (w/w) (Ameye et al., 2003).

2.2 Insulin

Insulin is a polypeptide hormone secreted by the β-cells of the islets of Langerhans of the endocrine pancreas in response to increased blood glucose levels. Insulin is synthesised by

the ribosomes bound to the rough endoplasmatic reticulum. The first precursor of insulin is preproinsulin. It contains a hydrophobic signal peptide at the amino terminus, which facilitates the transport of preproinsulin through the cell membranes of the endoplasmatic reticulum. Within the lumen of the endoplasmatic reticulum the signal peptide is cleaved by proteases to produce proinsulin. Proinsulin is transported to the Golgi complex where it is converted to insulin. Proinsulin consists of three segments: the A chain and B chain which are linked to each other with a 'connecting-peptide' or C chain. Also two disulfide bonds connect the A and B chain and there is a third disulfide bond connecting 2 cystein residuals on the A chain. Through proteolytic cleavage of the C chain insulin is formed consisting of 51 amino acids and having a molecular weight of 5808 Da (Figure 3) (Rawn, 1989).

The insulin molecule contains many ionisable groups, due to the presence of 6 amino acid residues capable of attaining a positive charge and 10 amino acid residues capable of attaining a negative charge. Its isoelectric point is at pH 5.4 (Brange, 1987).

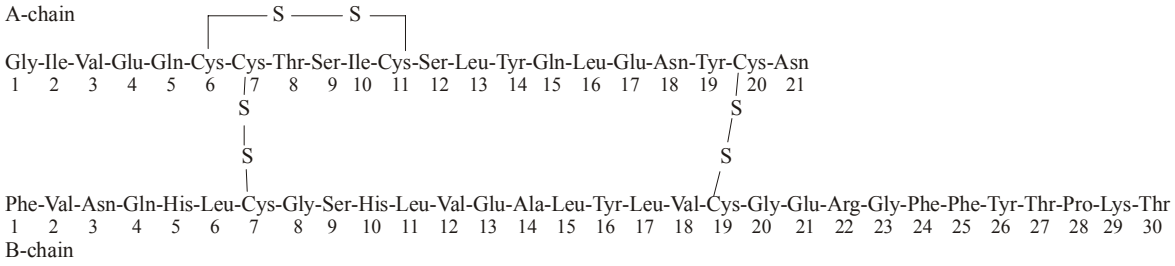


Figure 3. Amino acid sequence of human insulin. (Hoffman and Ziv, 1997)

The basal insulin secretion from the β-cells of the islets of Langerhans is approximately 1 IU/h. Stimulation of the insulin secretion as consequence of the digestion of food results in a release of 25-50 IU a day. Inadequate insulin secretion results in diabetes mellitus (Hoffman and Ziv, 1997). Two different types can be distinguished (Rawn, 1989):

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) occurs as a consequence of inadequate insulin production due to the inability to cleave proinsulin or to a genetic predisposition to an autoimmune destruction of the insulin-producing β-cells of the islets of Langerhans. The onset of IDDM usually occurs in early childhood and before the age of 20

and is characterised by hyperglycemia and ketoacidosis. Patients suffering from IDDM can effectively be treated with insulin.
Type II diabetes or non-insulin-dependent diabetes mellitus (NIDDM) generally appears in adults over the age of 40. NIDDM is characterised by a decreased production of insulin in the β-cells as well as by insulin resistance of various tissues. Diet control and physical exercise can usually control NIDDM and treatment with insulin is only required in extreme cases.

For 60 years, insulin was obtained by extraction from the pancreas of cows, pigs or sheep in sufficient quantities to cover the therapeutic needs. Bovine and porcine insulin were very impure and purification by repeated crystallisation, HPLC or ion-exchange chromatography was required. Insufficient purification resulted in immunological reactions to the impurities or the insulin molecule itself, limiting the use of these compounds. From an immunogenetic point of view, significant progress was made with the production of human insulin in the early 1980s using recombinant DNA technology or using an enzymatic reaction whereby the alanine residue at position B30 of pork insulin was replaced with a threonine residue (Trehan and Ali, 1998; Brange and Vølund, 1999).

2.3 Calcitonin

A second polypeptide hormone used in the research project was calcitonin consisting of 32 amino acids and having a molecular weight of approximately 3500 Da. It is characterised by a disulfide bridge between the cysteine residues at position 1 and 7 and a proline amide moiety at the C-terminus (Figure 4). The number of amino acid residues and the structure of the chain ends are identical in all calcitonins and appear to be essential for the biological activity. Salmon calcitonin and eel calcitonin are more potent in mammals, especially in man, in comparison with human or other mammalian calcitonins (Azria, 1989).

Calcitonin is secreted by the parafollicular (C) cells of the thyroid gland and its major physiological role is to control the calcium concentration as well as its metabolism in the body. It is responsible for the reduction of the amount of calcium in the blood stream as it increases the rate of calcium clearance from the kidneys. It also reduces the amount of calcium excreted by the bone, by inhibiting the osteoclast activity (decreased bone

resorption). Finally, it decreases the amount of calcium that can be absorbed from the small intestines. Its production is inhibited when the calcium concentration is decreased beyond normal levels and the parathyroid hormone is then secreted to promote the opposite reactions in the body of calcitonin. Both hormones act to maintain a normal concentration of calcium in the blood stream.

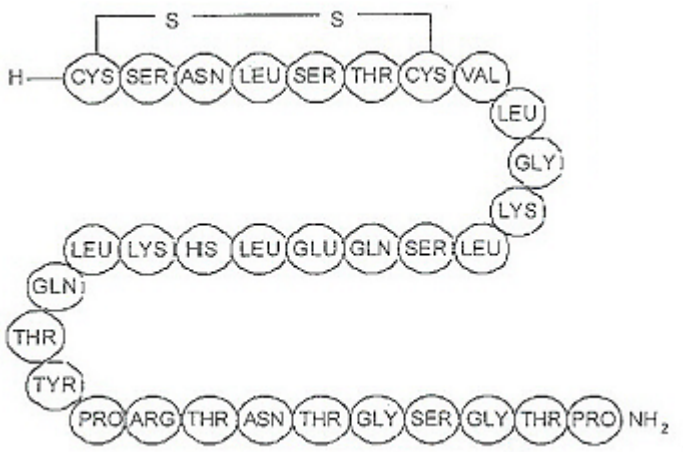


Figure 4. Amino acid sequence of salmon calcitonin. (Torres-Lugo and Peppas, 2000)

Due to their ability to reduce osteoclast activity, calcitonins are commonly used in the treatment of bone diseases such as Paget's disease, hypercalcemia and osteoporosis (Torres-Lugo and Peppas, 2000).

2.4 References

Ahuja A., Khar R.K., Ali J., Mucoadhesive drug delivery systems. Drug Dev. Ind. Pharm. 23 (1997) 489-515.

Ameye D., Remon J.P., Foreman P., Richardson P., Bioadhesive composition comprising a polysaccharide and a polycarboxylated polymer. PCT Patent WO 03/063839, August 7, 2003.

Azria M., The calcitonins: Introduction. In: Pittet R., Kälin H.B. (Eds.), The calcitonins: Physiology and pharmacology, Basel, Switzerland, 1989, 1-19.

Brange J., Galenics of Insulin. The physico-chemical and pharmaceutical aspects of insulin and insulin preparations. Springer-Verlag, Berlin, Germany, 1987.

Brange J., Vølund A., Insulin analogues with improved pharmacokinetic profiles. Adv. Drug Del. Rev. 35 (1999) 307-335.

Byars J.A., Jet cooking of waxy rice starch: solution rheology and molecular weight degradation of amylopectin. Cereal Chemistry 80 (2003) 87-90.

Hoffman A., Ziv E., Pharmacokinetic considerations of new insulin formulations and routes of administration. Clin. Pharmacokinet. 33 (1997) 285-301.

Newman A.W., Mueller R.L., Vitez I.M., Kiesnowski C.C., Starches and starch derivatives. In: Swarbrick J., Boylan J.C. (Eds.), Encyclopedia of pharmaceutical technology, Marcel Dekker Inc., New York, 2002, 2574-2581.

Rawn J.D., Glycogen metabolism, gluconeogenesis, pentose phosphate pathway: Insulin lowers the concentration of glucose in the blood. In: Patterson N. (Ed.), Biochemistry, Burlington, North Carolina, 1989, 395-398.

Singla A.K., Chawla M., Singh A., Potential applications of carbomer in oral mucoadhesive controlled drug delivery systems: a review. Drug Dev. Ind. Pharm. 26 (2000) 913-924.

Torres-Lugo M., Peppas N.A., Transmucosal delivery systems for calcitonin: a review. Biomaterials 21 (2000) 1191-1196.

Trehan A., Ali A., Recent approaches in insulin delivery. Drug Dev. Ind. Pharm. 24 (1998) 589-597.

Wade, A., Weller, J.P., Handbook of Pharmaceutical Excipients. The pharmaceutical press, London, 1994, 491-493.

Whistler R.L., Daniel J.R., Molecular structure of starch. In: Whistler R.L., BeMiller J.N., Paschall E.F. (Eds.), Starch: chemistry and technology, Academic press Inc., New York, 1984, 153-182.

Young A.H., Fraction of starch. In: Whistler R.L., Bemiller J.N., Paschall E.F. (Eds.), Starch: chemistry and technology, Academic Press Inc., New York, 1984, 249-283.

Chapter 3 Influence of bulk density on nasal bioavailability of insulin

3.1 Introduction

After nasal delivery of powder formulations based on a physical mixture of Drum Dried Waxy Maize starch and Carbopol® 974P (ratio: 90/10) (DDWM/C 974P 90/10) and on a spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75) (SD 25/75) an absolute nasal bioavailability of 14 % and 18 %, respectively, was found in rabbits (Callens et al., 2000, 2003a). The higher bioavailability of the latter was related to the higher viscosity and elasticity of the powder after dispersion in nasal mucus (Callens et al., 2003b).

Besides the powder characteristics the nasal bioavailability will depend on the deposition pattern of the powder formulation in the nasal cavity: anterior deposition of a formulation promotes the absolute bioavailability (Harris et al., 1986; Illum et al., 1987; Ridley et al., 1995; Callens, 2002).

The aim of the next experiment was to evaluate if the nasal bioavailability depended on the spray pattern of the powder. Callens (2002) determined the spray pattern of powder formulations based on DDWM/C 974P 90/10 and SD 25/75. For the latter a longer spray time was required to completely empty the powder from the nasal delivery device. The angle at which the particles were sprayed and the mean maximum and minimum width of the cross-section of the powder cloud were larger compared to the DDWM/C 974P powder. These differences were due to the higher bulk density and smaller particle size of SD 25/75, resulting in a compact powder having a higher resistance to airflow.

Bond et al. (1985) and Newman et al. (1987) showed that the cone angle of a nasal adaptor determined the width of the spray pattern and hence the deposition area in the nasal cavity. Changing the cone angle from 60 to 35 or 30° resulted in a posterior deposition in the nasal cavity and in a faster clearance rate of the formulation because of the higher deposition in the ciliated area. The smaller spray angle of the DDWM/C 974P 90/10 particles resulted in a narrow cross-section of the particle cloud (Callens, 2002) and might contribute to a posterior deposition in the nasal cavity and hence to the lower bioavailability obtained after nasal

delivery. On the other hand due to its lower bulk density and larger powder volume a larger area of the nasal epithelium might be covered with powder.
As the density of the formulation determined the spray pattern from the delivery device, the objective of the following experiment was to investigate the relation between the powder bulk density and nasal bioavailability of insulin using the rabbit as an animal model. The powder bulk density of the powder formulation was modified by changing the solid fraction of the dispersion (prior to freeze-drying) and by changing the freezing rate during the freeze-drying cycle.

3.2 Materials and methods

3.2.1 Materials

Actrapid® HM 100 (100 IU/ml) (human monocomponent insulin) was obtained from Novo-Nordisk (Bagsvaerd, Denmark). The spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75, w/w) (SD 25/75) (batch number: 10851: 141-1) was prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Drum Dried Waxy Maize starch (DDWM) and Carbopol® 974P (C 974P) were supplied by Eridania-Béghin Say Cerestar (Vilvoorde, Belgium) and Noveon (Cleveland, Ohio, USA), respectively. All other chemicals were of analytical grade.

3.2.2 Insulin formulations

3.2.2.1 Insulin solution for intravenous administration

An insulin solution of 0.8 IU/ml was prepared by diluting Actrapid® HM 100 in a phosphate buffered saline solution (PBS, pH 7.4) (2.38 g $Na_2HPO_4.2H_2O$, 0.19 g KH_2PO_4 and 8.0 g NaCl per liter distilled water) of which 0.5 ml was administered intravenously to rabbits (n = 10).

3.2.2.2 Nasal powder formulations

Dispersions containing a physical mixture of DDWM and C 974P (ratio: 95/5, w/w) (DDWM/C 974P 95/5) were prepared in a concentration of 2.0, 5.0 and 8.5 % (w/w), whereas dispersions containing a spray-dried mixture of Amioca® starch and C 974P (ratio: 25/75, w/w) (SD 25/75) were prepared in a concentration of 1.0, 2.5 and 7.0 % (w/w). To prepare the dispersions containing 2.0 and 5.0 % DDWM/C 974P 95/5, 1.0 g powder was dispersed in 10.0 ml distilled water and to prepare the dispersions containing 1.0 and 2.5 % SD 25/75, 1.0 g powder was dispersed in 30.0 ml distilled water. To prepare the dispersions containing 8.5 % DDWM/C 974P 95/5 and 7.0 % SD 25/75, 1.0 g powder was hydrated with 1.0 and 2.0 ml distilled water, respectively. After neutralisation of the dispersions containing DDWM/C 974P 95/5 and SD 25/75 to pH 7.4 with 0.2 M and 2.0 M NaOH, respectively, the insulin solution (Actrapid® HM 100) was added to obtain a final concentration of 1 IU insulin per mg powder. Finally distilled water was added until the desired concentration was obtained. The selection of the solid fractions of the dispersions was based on the consistency of the dispersions: dispersions having the lowest solid fraction had a liquid-like structure, dispersions with the intermediate solid fraction had a gel-like structure and dispersions with the highest solid fraction was the highest which could be prepared.

The dispersions were freeze-dried in vials using an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersions were frozen to 228 K within 175 min at 1000 mbar. Additionally, the dispersions containing 5.0 % DDWM/C 974P 95/5 and 2.5 % SD 25/75 were frozen to 228 K within 30 min and 350 min. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (283 K) and reduced pressure (0.1 - 0.2 mbar) for 7 h. After freeze-drying the powder was sieved (63 µm) at low relative humidity (20 %) and ambient temperature. The fraction below 63 µm was stored in a desiccator at 4 - 8 °C until use.

3.2.3 Nasal delivery device

The powders were nasally administered using an experimental device (Figure 1) composed of a polyethylene tube (inner diameter: 1.5 mm, length: 5.5 cm) (Medisize, Hillegom, The Netherlands), valve and polypropylene syringe (10 ml with Luer-Lok® needle holder). After closing the valve 7.8 ml air was compressed to 1 ml (generating an internal pressure of

± 2.5 bar). Then, a polyethylene tube filled with the powder formulation was connected to the syringe. The powder formulation was sprayed into the nostrils of the rabbits by opening the valve, releasing the compressed air. This experimental device was based on the system developed by Sørensen (1991).

Figure 1. Picture of experimental delivery device and of nasal powder delivery to rabbits.

3.2.4 Nasal bioavailability

New Zealand white rabbits (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available ad libitum. They were sedated with an intramuscular injection of 0.05 ml/kg Combistress® (Kela Laboratoria, Hoogstraten, Belgium). The rabbits received 0.4 IU insulin intravenously. The powder was administered intranasally using polyethylene tubes filled with 5.0 mg of the DDWM/C 974P 95/5 powder formulation or 10.0 mg of the SD 25/75 powder formulation. 10 mg powder (equivalent to 10 IU insulin) was administered in each nostril. In case of DDWM/C 974P 95/5 2 tubes filled with 5.0 mg powder were delivered into each nostril. The delivery device was filled under conditions of low relative humidity (20 %) and ambient temperature. Blood samples were collected from the ear veins at -5, 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after intravenous administration and at -5, 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min after nasal delivery of the powders. The samples were centrifuged (700g, 5 min) and the sera were frozen at –20 °C until RIA-analysis (Coat-A-Count® kit, DPC, Humbeek, Belgium). The radioactivity of the samples was quantified using a Cobra gamma counter (Canberra Packard Benelux, Zellik, Belgium). The individual serum concentration-time profiles were analysed using MW/Pharm version 3.15 (Medi-ware, Utrecht, The Netherlands). For calculation of the absolute biovailability (BA) the weight of the rabbits and the concentration of insulin in the powder formulations were

taken into account. The maximum serum insulin concentrations (C_{max}) and t_{max} values were determined from the individual serum concentration-time profiles.

- *Statistical analysis*

The influence of the solid fraction and the freezing rate on absolute nasal bioavailability, C_{max} and t_{max} of insulin was analysed using a one-way ANOVA. The data and residuals were tested for normal distribution using the Kolmogorov-Smirnov test and the homogeneity of variances was tested using the Levene's test. Multiple comparisons were performed by a Scheffé test using $P < 0.05$ as significance level. The computer software SPSS version 11.0 was used for statistical analysis of the data.

3.2.5 Determination of bulk density

The apparent powder bulk density (defined as the ratio of the powder weight over its bulk volume) was determined in the polyethylene tubes of the nasal delivery device filled with 5.0 mg of the freeze-dried DDWM/C 974P 95/5 mixture or 10.0 mg of the freeze-dried SD 25/75 mixture. Results are presented as a mean value ± standard deviation (n = 10).

- *Statistical analysis*

The influence of the solid fraction and the freezing rate on the powder bulk density was analysed using a one-way ANOVA followed by a Scheffé test using $P < 0.05$ as significance level. The data and residuals were tested for normal distribution using the Kolmogorov-Smirnov test and the homogeneity of variances was tested using the Levene's test. If the distribution of the data or residuals was not normal or the variances were not homogeneous, the data were transformed to their logarithm. The computer software SPSS version 11.0 was used for statistical analysis of the data.

3.2.6 Determination of particle size

The particle size of the powder formulations was determined by laser diffraction (Mastersizer S, Malvern Instruments, Worcestershire, UK), using Miglyol 812N

(caprylic/capric acid triglycerides) (Sasol, Witten, Germany) as dispersion medium. The volume median diameter (VMD) was calculated.

3.2.7 Moisture content

The moisture content was determined using a Mettler DL 35 Karl Fischer titrator (Mettler-Toledo, Beersel, Belgium). Hydranal®-Composite 2 (Riedel-de Haën Laborchemicalien, Seelze, Germany) was used as titrant and anhydrous methanol (Riedel-de Haën Laborchemicalien, Seelze, Germany) as reaction medium. Calibration and verification of the titrant (theoretical 2 mg H_2O/ml) was performed with distilled water. Results are presented as a mean value ± standard deviation (n = 3).

3.3 Results and discussion

To investigate the influence of powder bulk density on the nasal bioavailability of insulin using rabbits as animal model, the density of DDWM/C 974P 95/5 and SD 25/75 was modified by varying the solid fraction of the dispersion (prior to freeze-drying) and by changing the freezing rate.

The bulk density, particle size and moisture content of the powder formulations having different solid fractions are shown in Table 1. Significantly higher bulk densities ($P \leq 0.01$) were obtained when the solid fraction of the freeze-dried dispersion was increased. This increase in bulk density was not related to the volume median diameter as the different powders had a similar particle size. The differences in bulk density can be explained by the formation of smaller ice crystals during freezing. When processing a dispersion with a higher solid fraction, smaller ice crystals are formed, yielding a freeze-dried cake with smaller pores after sublimation of the ice (Pikal, 1991).

Table 1. Influence of the solid fraction of the dispersion (prior to freeze-drying) on powder bulk density, volume median diameter (VMD) and residual moisture content of powders containing DDWM/C 974P 95/5 or SD 25/75. The dispersions were frozen to 228 K within 175 min.

Solid fraction (w/w)	Bulk density (mg/µl)	VMD (µm)	Residual moisture (%)
DDWM/C 974P 95/5			
2.0 %	0.028 ± 0.002	69.0	5.8 ± 0.2
5.0 %	0.058 ± 0.004 [a]	62.1	5.0 ± 0.1
8.5 %	0.083 ± 0.008 [a,b]	49.2	3.6 ± 0.3
SD 25/75			
1.0 %	0.116 ± 0.009	55.8	9.9 ± 0.9
2.5 %	0.165 ± 0.016 [c]	51.9	7.5 ± 1.0
7.0 %	0.184 ± 0.015 [c,d]	41.9	6.8 ± 1.0

[a] Significantly higher than 2.0 % (w/w) dispersion ($P \leq 0.001$)

[b] Significantly higher than 5.0 % (w/w) dispersion ($P \leq 0.001$)

[c] Significantly higher than 1.0 % (w/w) dispersion ($P \leq 0.001$)

[d] Significantly higher than 2.5 % (w/w) dispersion ($0.01 \geq P > 0.001$)

The powder bulk density, particle size and moisture content of the different powder formulations frozen at different freezing rates are shown in Table 2. Varying the freezing rate had a limited effect on the powder bulk density. Only freezing at the highest freezing rate resulted in a significantly higher bulk density ($P \leq 0.001$) of the SD 25/75 powder compared to slower freezing rates. Fast freezing resulted in the formation of smaller ice crystals due to a higher degree of supercooling (Dawson and Hockley, 1991) resulting in smaller pores of the freeze-dried cake after sublimation. Possibly the effect of the size of the ice crystals on the powder bulk density is more pronounced at a lower solid fraction because of the higher water content.

The solid fractions of the dispersions and freezing rates used are production limits, hence the powder bulk densities obtained can also be considered as the limits. Although the bulk density varied within a small range, these differences could be essential as the nasal cavity of rabbits has only a small volume (6 ml) and a limited surface area (61 cm^2) (Gizurarson, 1990). As a result small changes of the powder density might result in significant differences

Table 2. Influence of freezing rate on powder bulk density, volume median diameter (VMD) and residual moisture content of powder formulations prepared by freeze-drying dispersions containing 5.0 % DDWM/C 974P 95/5 or 2.5 % SD 25/75.

Freezing rate to 228 K within	Bulk density (mg/µl)	VMD (µm)	Residual moisture (%)
DDWM/C 974P 95/5			
30 min	0.060 ± 0.004	71.2	6.41 ± 0.21
175 min	0.058 ± 0.004	62.1	5.04 ± 0.12
350 min	0.063 ± 0.006	47.7	4.72 ± 0.15
SD 25/75			
30 min	0.202 ± 0.010	61.3	3.55 ± 0.24
175 min	0.165 ± 0.016 [a]	51.9	7.54 ± 0.99
350 min	0.160 ± 0.014 [a]	48.5	3.72 ± 0.22

[a] Significantly lower than freezing to 228 K within 30 min ($P \leq 0.001$)

in the area of nasal epithelium covered with powder particles. A larger fraction of the nasal epithelium covered with particles will occur after administration of low bulk density powders (because of their larger volume) and could contribute to a higher nasal bioavailability. Comparison of two nasal sprays, each containing 2 % didecanoyl-L-α-phosphatidylcholine and 200 IU or 500 IU insulin/ml, in healthy volunteers demonstrated that a higher absolute bioavailability was obtained after nasal administration of the 200 IU/ml formulation (13.2 % versus 8.8 % for the 500 IU/ml formulation). This was related to the greater surface area, which was covered by the 200 IU/ml formulation due to its larger dose volume (0.25 ml versus 0.1 ml for the 500 IU/ml formulation) (Jacobs et al., 1993). However, it must be emphasised that a more extensive spreading over the nasal epithelium does not guarantee a higher bioavailability as the distribution of the formulation between anterior and posterior part of the nose remains an important parameter for absorption (Kublik and Vidgren, 1998).

The results of the absolute bioavailability, C_{max} and t_{max} obtained after nasal delivery of DDWM/C 974P 95/5 and SD 25/75 powder formulations to rabbits are shown in Table 3 and 4.

Table 3. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) after nasal delivery to rabbits of powder formulations (1 IU insulin/mg) obtained by freeze-drying DDWM/C 974P 95/5 or SD 25/75 dispersions at different solid fractions. The dispersions were frozen to 228 K within 175 min.

Solid fraction (w/w)	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
DDWM/C 974P 95/5				
2.0 %	6.8 ± 1.7	449.8 ± 166.9	22.1 ± 4.3	7
5.0 %	9.2 ± 3.0	497.1 ± 184.6	23.3 ± 5.5	8
8.5 %	5.8 ± 1.9 [a]	353.0 ± 144.6	23.7 ± 6.8	8
SD 25/75				
1.0 %	17.4 ± 5.3	588.6 ± 112.5	44.4 ± 13.4	7
2.5 %	19.2 ± 5.3	681.4 ± 246.6	50.9 ± 7.4	8
7.0 %	11.1 ± 3.0 [b]	455.5 ± 99.4	39.9 ± 6.5	7

[a] Significantly lower than 5.0 % (w/w) dispersion ($0.05 \geq P > 0.01$)

[b] Significantly lower than 2.5 % (w/w) dispersion ($0.05 \geq P > 0.01$)

Table 4. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) after nasal delivery to rabbits of powder formulations (1 IU insulin/mg) obtained by freeze-drying dispersions containing 5.0 % DDWM/C 974P 95/5 or 2.5 % SD 25/75 at different freezing rates.

Freezing to 228 K within	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
DDWM/C 974P 95/5				
30 min	5.4 ± 2.6	281.1 ± 192.8	29.9 ± 5.3	7
175 min	9.2 ± 3.0	497.1 ± 184.6	23.3 ± 5.5	8
350 min	8.2 ± 3.0	478.7 ± 163.0	22.4 ± 4.4	8
SD 25/75				
30 min	18.2 ± 3.7	708.4 ± 210.2	41.5 ± 9.3	7
175 min	19.2 ± 5.3	681.4 ± 246.6	50.9 ± 7.4	8
350 min	22.4 ± 3.3	852.4 ± 84.0	40.9 ± 11.7	6

Although significant differences in absolute bioavailability were observed after nasal delivery to rabbits, no influence of powder bulk density on the absolute bioavailability of insulin was found. When increasing the solid fraction of the dispersion an increase in powder bulk density was seen (Table 1). However, the bioavailability after nasal delivery was similar.

Only for powders prepared by freeze-drying the dispersions containing 8.5 % DDWM/C 974P 95/5 and 7.0 % SD 25/75 a significant decrease in area under the curve ($P < 0.05$) was seen, although their C_{max} and t_{max} values were not significantly ($P > 0.05$) different.

Based on these data, it was indirectly demonstrated that differences in the spray pattern of nasal powders due to variations in the powder bulk density have no influence on the nasal insulin bioavailability in rabbits.

3.4 Conclusions

The bulk density was mainly influenced by the solid fraction of the dispersion prepared prior to freeze-drying. The bulk density of the powder formulations had no influence on the nasal bioavailability of insulin in rabbits.
From this study, it was concluded that the procedure for preparation of the nasal powder formulations was a robust production method.

3.5 References

Bond S.W., Hardy J.G., Wilson C.G., Deposition and clearance of nasal sprays. In: Aiache J.M., Hirtz J. (Eds.), Proceedings volume I, Biopharmaceutics, 1985, 93-98.

Callens C., Remon J.P., Evaluation of starch-maltodextrin-Carbopol® 974P mixtures for the nasal delivery of insulin in rabbits. J. Control. Rel. 66 (2000) 215-220.

Callens C., Ontwikkeling van een platform voor de nasale toediening van peptiden. Doctoral thesis, Ghent University, Belgium, 2002.

Callens C., Pringels E., Remon J.P., Influence of multiple nasal administrations of bioadhesive powders on the insulin bioavailability. Int. J. Pharm. 250 (2003a) 415-422.

Callens C., Ceulemans J., Ludwig A., Foreman P., Remon J.P., Rheological study on mucoadhesivity of some nasal powder formulations. Eur. J. Pharm. Biopharm. 55 (2003b) 323-328.

Dawson P.J., Hockley D.J., Scanning electron microscopy of freeze-dried preparations: Relationship of morphology to freeze-drying parameters. Develop. Biol. Standard 74 (1991) 185-192.

Gizurarson S., Animal models for intranasal drug delivery studies: A review article. Acta Pharm. Nord 2 (1990) 105-122.

Harris A.S., Nilsson I.M., Wagner Z.G., Alkner U., Intranasal administration of peptides - nasal deposition, biological response and absorption of desmopressin. J. Pharm. Sci. 75 (1986) 1085-1088.

Illum L., Jorgensen H., Bisgaard H., Krogsgaard O., Rossing N., Bioadhesive microspheres as a potential nasal drug delivery system. Int. J. Pharm. 39 (1987) 189-199.

Jacobs M.A.J.M., Schreuder R.H., Jap-A-Joe K., Nauta J.J., Andersen P.M., Heine R.J., The pharmacodynamics and activity of intranasally administered insulin in healthy male volunteers, Diabetes 42 (1993) 1649-1655.

Kublik H., Vidgren M.T., 1998. Nasal delivery systems and their effect on deposition and absorption. Adv. Drug Del. Rev. 29 (1998) 157-177.

Newman S.P., Morén F., Clarke S.W., Deposition pattern of nasal sprays in man. Rhinology 26 (1987) 111-120.

Pikal M.J., Freeze-drying of proteins. Part I: Process design. Pharm. Techn. Int. 1 (1991) 37-40.

Ridley D., Perkins A.C., Washington N., Wilson C.G., Wastie M.L., Oflynn P., Blattman A., Ponchel G., Duchene D., The effect of posture on nasal clearance of bioadhesive starch microspheres. STP Pharma Sciences 5 (1995) 442-446.

Sørensen A., Animal models for buccal and nasal delivery studies. In: Duchêne D. (Ed.), Buccal and nasal administration as an alternative to parenteral administration. European Symposium, Paris, 10-11 Dec, Editions de Santé (1991) 162-173.

Chapter 4 Addition of divalent cations to spray-dried Amioca® starch/Carbopol®

Chapter 4.1 Influence of addition of divalent cations to spray-dried Amioca® starch/Carbopol® on nasal absorption of peptides

4.1.1 Introduction

To enhance the nasal absorption of peptides Callens et al. (2000, 2003) used powder formulations based on neutralised mixtures of starch and poly(acrylic acid). With a formulation composed of spray-dried Amioca® starch/Carbopol® 974P (ratio: 25/75) and containing 1 IU insulin/mg an absolute bioavailability of 18 % was obtained in rabbits (Callens, 2003). The aim of the present study was to investigate if the neutralising agent had an influence on the absorption of peptides. Whereas Callens et al. (2000, 2003) only used sodium hydroxide to neutralise the polymer, metal inorganic compounds (calcium hydroxide or calcium carbonate) will also be evaluated in this study as neutralising agents.

In literature it has been described that divalent ions affected the drug release and mucoadhesion of poly(acrylic acid) polymers (Lee and Chien, 1996). Tablets composed of starch-acrylic acid graft copolymers partially neutralised with calcium or magnesium exhibited a slower theophylline release, a better mucoadhesion and a longer adhesion time to the gingiva of dogs compared to the sodium-neutralised formulation (Geresh et al., 2004). Adding calcium or magnesium ions to a polymethylmethacrylate sodium salt used as carrier for buccal drug delivery reduced the dissolution rate of the polymer without influencing the bioadhesive properties, thus prolonging the adhesion time in human volunteers (Cilurzo, 2005).

In the present study the influence of calcium incorporated in sodium salts of starch/Carbopol® mixtures was investigated on the nasal absorption of insulin and salmon calcitonin in rabbits.

4.1.2 Materials and methods

4.1.2.1 Materials

Actrapid® HM 100 (100 IU/ml) (human monocomponent insulin) and Miacalcic® (100 IU/ml) were obtained from Novo-Nordisk (Bagsværd, Denmark) and Novartis Pharma (Brussels, Belgium), respectively. Spray-dried Amioca® starch and a spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75, w/w) (SD 25/75) (batch number: 10851: 141-1) were prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Carbopol® 974P was supplied by Noveon (Cleveland, Ohio, USA). Calcium hydroxide ($Ca(OH)_2$) and calcium carbonate ($CaCO_3$) (density: 0.30 g/ml) were obtained from Sigma-Aldrich (Bornem, Belgium) and Federa (Brussels, Belgium), respectively. All other chemicals were of analytical grade.

4.1.2.2 Preparation of formulations

4.1.2.2.1 Insulin solution for intravenous administration

An insulin solution of 0.8 IU/ml was prepared by diluting Actrapid® HM 100 in a phosphate buffered saline solution (PBS, pH 7.4) (2.38 g $Na_2HPO_4.2H_2O$, 0.19 g KH_2PO_4 and 8.0 g NaCl per liter distilled water) of which 0.5 ml was administered intravenously to rabbits (n = 10).

4.1.2.2.2 Calcitonin solution for intravenous administration

A salmon calcitonin solution of 20 IU/ml was prepared by diluting Miacalcic® (100 IU/ml) in a phosphate buffered saline solution (pH 7.4) of which 0.5 ml was administered intravenously to rabbits (n = 2).

4.1.2.2.3 Nasal powder formulations

Physical mixtures of SD 25/75 combined with $Ca(OH)_2$ or $CaCO_3$ were made in a 90/10 (w/w) ratio. To investigate the influence of the Amioca® starch/Carbopol® 974P versus

$Ca(OH)_2$ ratio on the absorption of insulin, physical mixtures at a ratio of 90/1, 90/20 and 90/30 (w/w) were also prepared. To investigate the influence of spray-drying, a physical mixture of Amioca® starch (spray-dried), Carbopol® 974P (ratio: 25/75, w/w) (PM 25/75) and $Ca(OH)_2$ was made, at a ratio of 90/10 (w/w) between PM 25/75 and $Ca(OH)_2$. The mixtures of SD 25/75 and PM 25/75 were used as reference.

A 500 mg powder blend was dispersed in 15 ml distilled water followed by neutralisation until pH 7.4 with 2.0 M NaOH (Table 1). Then the insulin solution (Actrapid® HM 100) or salmon calcitonin solution (Miacalcic®) was added in order to obtain a final concentration of 1 IU insulin or 2 IU salmon calcitonin per mg powder, respectively.

Table 1. Amount of NaOH 2.0 M required to reach pH 7.4 after dispersion of 500 mg powder formulation into distilled water.

Formulation	ml NaOH 2.0M
SD 25/75	2.15
(SD 25/75)/$Ca(OH)_2$ 90/1	2.0
(SD 25/75)/$Ca(OH)_2$ 90/10	1.30
(SD 25/75)/$Ca(OH)_2$ 90/20	0.7
(SD 25/75)/$Ca(OH)_2$ 90/30	0.1
(SD 25/75)/$CaCO_3$ 90/10	1.50
PM 25/75	2.0
(PM 25/75)/$Ca(OH)_2$ 90/10	1.35

To obtain a powder, the aqueous dispersion was freeze-dried using an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to 228 K within 175 min at 1000 mbar. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (283 K) and reduced pressure (0.1 - 0.2 mbar) for 7 h. After freeze-drying the powder was sieved (63 μm) at low relative humidity (20 %) and ambient temperature. The fraction below 63 μm was stored in a desiccator at 4 - 8 °C until use.

4.1.2.3 Nasal bioavailability study

New Zealand white rabbits (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available ad libitum. They were sedated with an intramuscular injection of 0.05 ml/kg Placivet® (Codifar, Florida, USA). The rabbits received 0.4 IU insulin or 10 IU salmon calcitonin intravenously. 10 mg powder (equivalent to 10 IU insulin or 20 IU salmon calcitonin) was administered in each nostril using polyethylene tubes (Medisize, Hillegom, The Netherlands). The powder was released from the tubes using a syringe containing 1 ml compressed air (2.5 bar). This device was based on a system developed by Sørensen (1991). The tubes were filled under conditions of low relative humidity (20 %) and ambient temperature. Blood samples were collected from the ear veins at -5, 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after intravenous administration and at -5, 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min after nasal delivery of the powder formulations. The samples were centrifuged (700g, 5 min) and the sera were frozen at –20 °C until analysis. The samples containing insulin were analysed using RIA (Coat-A-Count® kit, DPC, Humbeek, Belgium). The radioactivity of the samples was quantified using a Cobra gamma counter (Canberra Packard Benelux, Zellik, Belgium). The samples with calcitonin were analysed using an Enzyme-Linked Immunosorbent kit (Active® Ultra-sensitive salmon calcitonin, Diagnostic Systems Laboratories, Texas, USA). The fluorescence measurements were carried out on a fluorometer (Wallac 1420 multilabel counter, PerkinElmer, Turku, Finland) at 450 nm.
The basal levels of calcitonin were determined by collecting blood samples from the ear veins at -5, 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min from an untreated rabbit (n = 1).
The individual serum concentration-time profiles were analysed using MW/Pharm version 3.15 (Medi-ware, Utrecht, The Netherlands). For calculation of the absolute biovailability (BA) the weight of the rabbits and the concentration of insulin or salmon calcitonin in the powder formulations were taken into account. For salmon calcitonin the basal levels were also taken into account. The maximum serum insulin concentrations (C_{max}) and t_{max} values were determined from the individual serum concentration-time profiles.

- *Statistical analysis*

The influence of the powder formulations on the absolute nasal bioavailability, C_{max} and t_{max} of insulin or salmon calcitonin was analysed using One-way ANOVA. The data and residuals

were tested for normal distribution using the Kolmogorov-Smirnov test and the homogeneity of variances was tested using the Levene's test. If the distribution of the data or residuals was not normal or the variances were not homogeneous, the data were transformed (logarithm, square root or power). Specific sets were compared using Contrast Analysis ($P < 0.05$). The software program SPSS version 11.0 was used for statistical analysis.

4.1.2.4 Rheological properties

The elasticity (G') and viscosity (G") of the powders were determined on a TA Instruments AR 1000-N Rheometer (Zellik, Belgium) after dispersion (10 %, w/w) in simulated nasal fluid (7.45 mg/ml NaCl, 1.29 mg/ml KCl and 0.32 mg/ml $CaCl_2$, Melon, 1968). The measurements were performed at 32 ± 0.5 °C using a cone of 4 cm with an angle of 1° and applying an oscillation stress of 1.4 Pa and a frequency of 0.1 Hz. The measurements were performed on SD 25/75 powders mixed with different concentrations of $Ca(OH)_2$, but without insulin.

4.1.2.5 Physical analysis

The physical analysis of the powder formulations was performed by Prof. Dr. Ronald Verbeeck *(Department of Dental Materials Science, Faculty of Medicine and Health Sciences, Ghent University)*.

The neutralised (SD 25/75)/$Ca(OH)_2$ powders were characterised by IR spectroscopy and X-ray diffractometry. IR spectra of the samples dispersed in KBr tablets were recorded between 400 and 4000 cm^{-1} with a resolution of 1 cm^{-1} using a Galaxy 6030 Fourier transform IR spectrophotometer (Mattson, Madison, WI, USA). X-ray powder diffraction patterns were recorded between 2 and 60° 2θ by step-scanning with a microprocessor-controlled diffractometer system (PW 1830, Philips, Almelo, The Netherlands). Ni-filtered copper Kα radiation was used with an automatic divergence slit (PW 1836) and a graphite monochromator. The step scanning was performed with an integration time of 4 sec at intervals of 0.02° (2θ). The measurements were performed on the nasal powder formulations without insulin.

4.1.2.6 TEER experiment

The TEER experiments were performed by Raf Mols and Prof. Dr. Patrick Augustijns *(Laboratory for Pharmacotechnology and Biopharmacy, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven).*

4.1.2.6.1 Materials

All chemicals used for culturing the Caco-2 cells were purchased from Cambrex (Verviers, Belgium). Cell culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 1 % MEM non-essential amino acids solution and 100 IU/ml penicillin-streptomycin. Transport medium (TM) consisted of Hanks' balanced salt solution (HBSS) containing 25 mM D-(+)-glucose (Sigma, St. Louis, MO) and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); pH was adjusted to 7.4 at 37 °C with NaOH 2.0 M (BDH, Poole, UK).

4.1.2.6.2 Cell culture

Caco-2 cells originating from a human colon adenocarcinoma were purchased from Cambrex Biosciences (Walkersville, MD). Caco-2 cells were cultivated in 75 cm^2 culture flasks at 37 °C in an atmosphere of 5 % CO_2 and 90 % relative humidity. Cells were passaged every 3 - 4 days (at 70 - 80 % confluence) at a split ratio of 1 - 7. Cells were negative for mycoplasma infection.

4.1.2.6.3 TEER experiments

For transport experiments, Caco-2 cells were plated at a density of 40 000 cells/cm^2 on Costar® Transwell membrane inserts (3 µm pore diameter, 12 mm diameter) (Corning, New York, USA). Confluence was reached within 3 - 4 days after seeding and the monolayers were used for the experiments 17 days post-seeding. Cell passage 42 was used in the experiments. Transepithelial electrical resistance-values (TEER values) were measured with an EndOhm Voltohmmeter (WPI, Aston, UK). TEER measurements are a means to check the

integrity of the monolayer and can reveal toxicity or an opening of tight junctions induced by the drug or excipients. Only monolayers with initial TEER values higher than 200 $\Omega.cm^2$ were used.

After rinsing the monolayers three times with calcium-free transport medium, the apical medium was removed and the cell culture inserts were transferred into wells containing 1.5 ml transport medium. 1 mg (target amount) of the powder formulation SD 25/75 or (SD 25/75)/$Ca(OH)_2$ 90/10 was spread over the cell surface. After incubation of the cells during 2 or 5 min, 500 µl calcium-free transport medium was added to the apical side of the cell monolayer. TEER values were recorded immediately after adding the calcium-free transport medium and also 15, 30, 45 and 60 min after having added the powders.

4.1.2.7 Liquid uptake rate

The liquid uptake was studied hydrodynamically. 50 mg powder was placed on the upper side of a filter connected to a reservoir filled with simulated nasal fluid (SNF) (7.45 mg/ml NaCl, 1.29 mg/ml KCl and 0.32 mg/ml $CaCl_2$, Melon, 1968). The measurements were performed at 32 ± 0.5 °C. The amount of SNF absorbed was determined volumetrically in function of time.

4.1.2.8 Transmission electron microscopy study of the nasal epithelium

The transmission electron microscopy was performed by Dorothea Van Limbergen and Prof. Dr. Claude Cuvelier *(Department of Pathology, Faculty of Medicine and Health Sciences, Ghent University).*

10 mg powder composed of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 was sprayed into each nostril of New Zealand white rabbits (3.0 ± 0.5 kg). 15 min after nasal administration the rabbits were sacrificed by exsanguination of the femoral arteries. Immediately after exsanguination the nasal cavity was excised and fixed in a 4 % paraformaldehyde/5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.5). After fixation the tissue was cut in small slices and washed during 12 h in a 0.1 M sodium cacodylate buffer (pH 7.5). Postfixation was performed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Then the tissue slices were dehydrated and embedded in Epon 812. Semi-thin (1 µm)

sections, stained with toluidin blue, were used for orientation of the tissue. Ultra-thin sections (60 nm) were stained with uranylacetate and lead nitrate and examined with a Zeiss TEM 900. Digital pictures were taken with a Camera JENOPTIC JENA laser optic system.

4.1.3 Results and discussion

4.1.3.1 Addition of divalent cations to spray-dried Amioca®/Carbopol®

Callens et al. (2003) demonstrated that nasal delivery of a powder formulation consisting of a neutralised mixture of spray-dried Amioca® starch/Carbopol® 974P (ratio: 25/75) resulted in an absolute bioavailability of 18 % in rabbits. Sodium hydroxide was used to neutralise the poly(acrylic acid) fraction, forming a sodium poly(acrylate). The aim of this study was to evaluate if Ca^{2+}-poly(acrylates) were able to enhance the nasal absorption of insulin. Calcium poly(acrylates) were produced in situ by dispersing a physical mixture of spray-dried Amioca® starch/Carbopol® 974P (ratio: 25/75) and $Ca(OH)_2$ (ratios: 90/1, 90/10, 90/20 and 90/30) into distilled water. The interaction between the carboxylate groups of poly(acrylic acid) (≈ pH 3.1) and $Ca(OH)_2$ and the neutralisation to pH 7.4 with NaOH resulted in a mixture of Na^+- and Ca^{2+}-carboxylate. The SD 25/75 formulation contained exclusively Na^+-carboxylate, while the (SD 25/75)/$Ca(OH)_2$ 90/30 formulation was mainly composed of Ca^{2+}-carboxylate.

The different nasal formulations were analysed by X-ray diffractometry to confirm the in situ formation of Ca^{2+}-carboxylates (Figure 1). The XRD patterns of the (SD 25/75)/$Ca(OH)_2$ powder formulations showed some broad bands comparable to the XRD pattern of the SD 25/75 mixture without $Ca(OH)_2$. Diffraction peaks corresponding to the most intense diffraction peaks characteristic of crystalline $Ca(OH)_2$ could not be detected. As the intense and sharp absorption peak of OH stretching vibration around 3643 cm^{-1}, which is typical for $Ca(OH)_2$, could also not be detected in the IR spectra of the (SD 25/75)/$Ca(OH)_2$ powder formulation, the physical analyses indicated that the entire $Ca(OH)_2$ content interacted with poly(acrylic acid). The IR spectra of the SD 25/75 powder formulations with and without $Ca(OH)_2$ showed an intense and relative broad band centred at about 1560 cm^{-1} and a less intense absorption at 1454 cm^{-1} from the asymmetric and symmetric carbonyl stretching

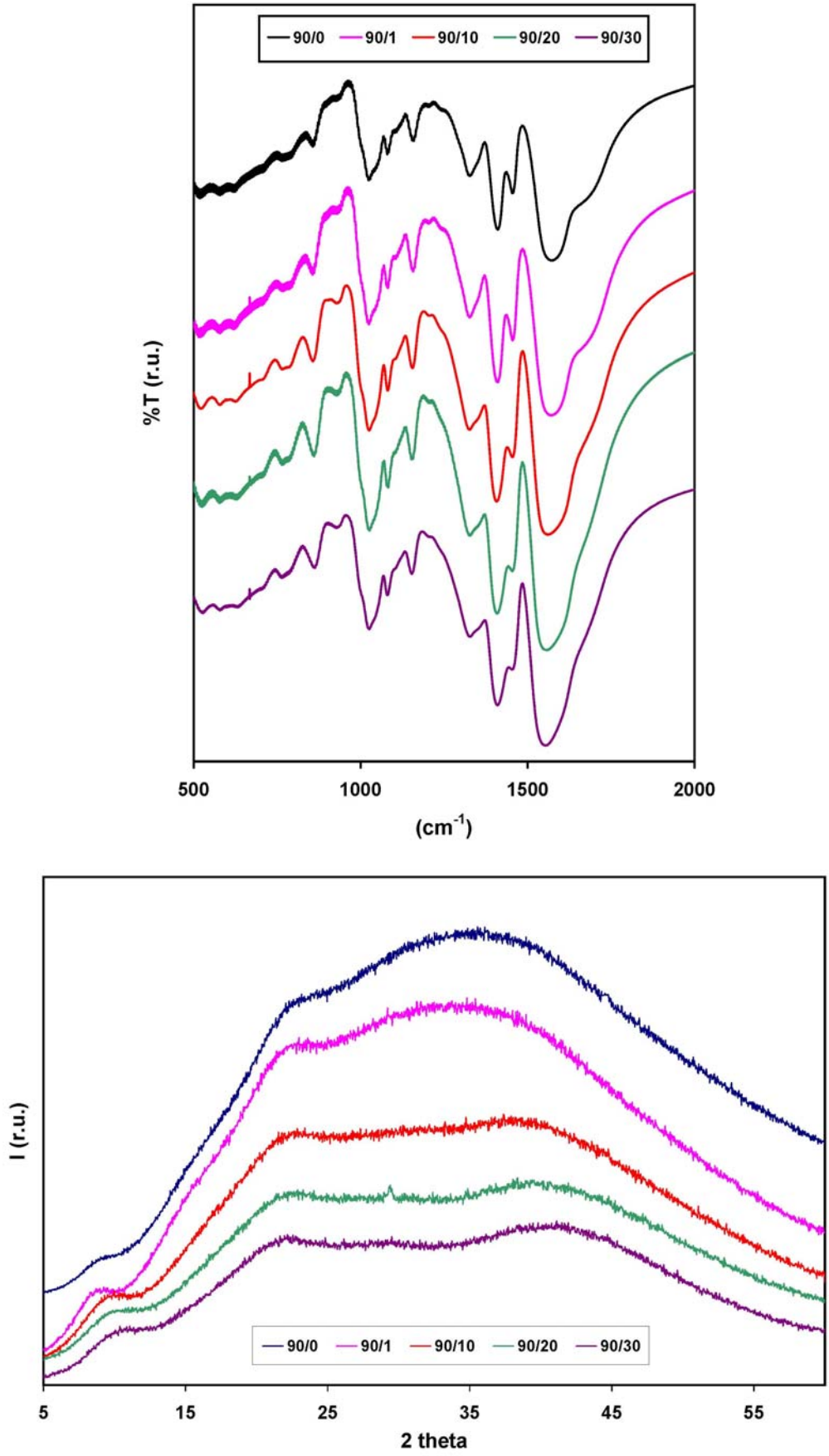


Figure 1. IR spectra (at the top) and X-ray diffraction patterns (at the bottom) of powder formulations containing (SD 25/75)/$Ca(OH)_2$ at different ratios.

vibration in poly(acrylate) salts (Crisp et al., 1974; Nicholson et al., 1988; Deb and Nicholson, 1999). Whereas the position of the absorption peak at 1454 cm^{-1} was independent of the amount of $Ca(OH)_2$ added, the centre of the band at 1560 cm^{-1} shifted with increasing $Ca(OH)_2$ concentration from 1575 cm^{-1} for the SD 25/75 mixture without $Ca(OH)_2$ (containing only sodium poly(acrylate)) to 1552 cm^{-1} for (SD 25/75)/Ca(OH) 90/30 (containing mainly calcium poly(acrylate)) (Figure 1). This indicated a gradual transition from an ionic structure to a chelating bidentate structure typical for calcium poly(acrylate) (Nicholson et al., 1988; Deb and Nicholson, 1999). In all spectra a weak but distinct absorption at 1680 cm^{-1} was observed which appeared as a shoulder on the 1560 cm^{-1} band. This absorption is due to the carbonyl vibration in carboxyl groups, indicating that some COOH-groups of poly(acrylic acid) were not neutralised (Crisp et al., 1974; Nicholson et al., 1988).

The fact that nearly all $Ca(OH)_2$ in the powder formulations participated in the neutralisation of Carbopol® was observed indirectly during the preparation of the nasal powders. Aqueous dispersions of powder blends containing more $Ca(OH)_2$ required less NaOH to reach pH 7.4, indicating that partial neutralisation of the carboxylic acid functions of Carbopol® occurred by $Ca(OH)_2$. Furthermore, from the amount of $Ca(OH)_2$ in the formulation and the amount of NaOH required to obtain pH 7.4, the free Ca^{2+}-ion concentration could be estimated and was found to be minimal (Table 2).

Table 2. Amount of Na^+ and Ca^{2+} bound to Carbopol® 974P and amount of free Ca^{2+}-ions in (SD 25/75)/$Ca(OH)_2$ powders calculated from the amount of $Ca(OH)_2$ present in the formulation and the amount of NaOH required to reach pH 7.4.

Formulation	mg Na^+ bound/ g polymer	mg Ca^{2+} bound/ g polymer	mg free Ca^{2+}/ g polymer
SD 25/75	263.1	-	-
(SD 25/75)/$Ca(OH)_2$ 90/1	252.1	9.5	-1.5
(SD 25/75)/$Ca(OH)_2$ 90/10	174.5	77.1	3.0
(SD 25/75)/$Ca(OH)_2$ 90/20	106.3	136.1	23.8
(SD 25/75)/$Ca(OH)_2$ 90/30	18.4	212.8	27.4

In the next experiment the absolute nasal bioavailability of insulin after administration of SD 25/75 physically mixed with $Ca(OH)_2$ at a ratio 90/10 was determined in rabbits. The addition of $Ca(OH)_2$ significantly increased the absolute nasal bioavailability ($P < 0.001$) and C_{max} ($P < 0.001$), while t_{max} significantly ($P < 0.001$) decreased (Table 3).

Table 3. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) after nasal delivery to rabbits of powders (1 IU insulin/mg) containing SD 25/75 physically mixed with $Ca(OH)_2$ at different ratios.

Formulation	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
[b] SD 25/75	19.2 ± 5.3	681 ± 247	50.9 ± 7.4	8
[b] (SD 25/75)/$Ca(OH)_2$ 90/1	24.6 ± 9.2	958 ± 484	44.5 ± 9.6	6
[a] (SD 25/75)/$Ca(OH)_2$ 90/10	29.0 ± 11.4***	1813 ± 828***	27.3 ± 3.4	8
[b] (SD 25/75)/$Ca(OH)_2$ 90/20	9.6 ± 2.5	956 ± 225	15.1 ± 2.9	7
[b] (SD 25/75)/$Ca(OH)_2$ 90/30	2.2 ± 0.7	181 ± 51	10.2 ± 4.6	8

The BA and C_{max} of set[a] was compared to set[b] using Contrast Analysis (*** = $P \leq 0.001$).

The influence of the ratio SD 25/75 versus $Ca(OH)_2$ (90/1, 90/10, 90/20 and 90/30) on bioavailability was also investigated. After nasal delivery of these powders to rabbits a gradual increase in absorption of insulin was observed when the $Ca(OH)_2$ content increased. A maximum was seen for the ratio 90/10. A further increase in $Ca(OH)_2$ content to a ratio of 90/30 resulted in a sudden drop of nasal insulin absorption (Figure 2, Table 3). Furthermore a linear relationship was obtained ($P < 0.001$, Linear Contrast Analysis) between $Ca(OH)_2$ concentration and t_{max}, this parameter decreased at higher calcium concentration. The ratio of Amioca® starch/Carbopol® 974P versus $Ca(OH)_2$ had an important influence on the extent of absorption enhancement of insulin.

In order to evaluate if a similar absorption enhancement could be obtained using another calcium salt, $CaCO_3$ was incorporated in the carrier containing SD 25/75 at a ratio 90/10. For this formulation a similar ($P > 0.05$) absolute bioavailability (26.1 ± 9.8 %), C_{max} (1288 ± 670 µIU/ml) and t_{max} (28.7 ± 4.6 min) was obtained compared to the formulation with $Ca(OH)_2$.

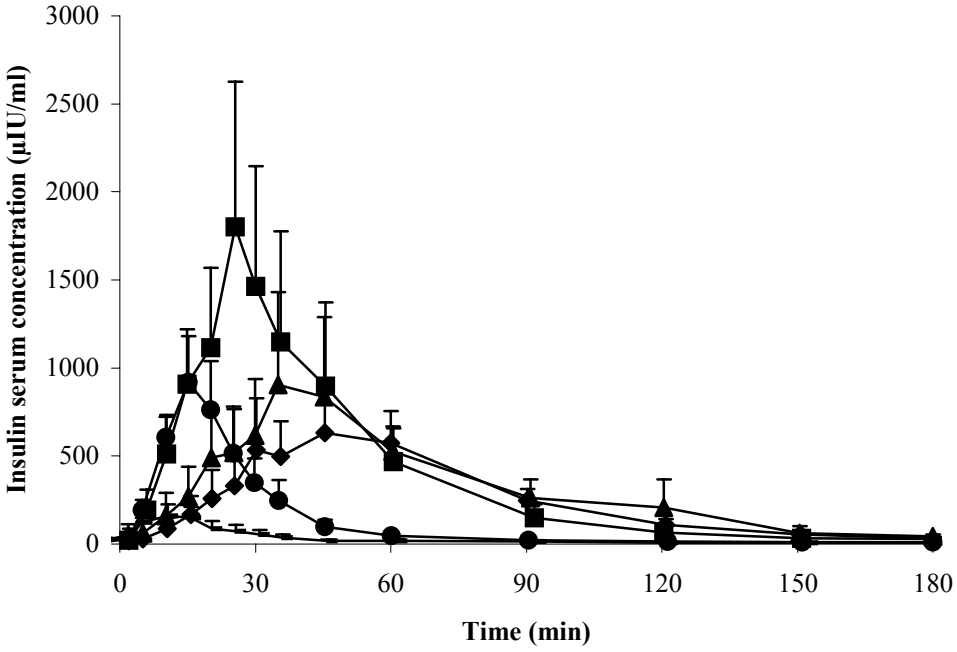


Figure 2. Insulin serum concentration-time profiles after nasal delivery to rabbits of powder formulations (1 IU insulin/mg) containing SD 25/75 physically mixed with $Ca(OH)_2$ at different ratios: (90/1 (▲), 90/10 (■), 90/20 (●) and 90/30 (-). The formulation based on SD 25/75 without $Ca(OH)_2$ (◆) was used as reference.

It is known that poly(acrylates) have the potential to bind calcium ions. They were able to prevent denaturation of proteins by enzymes that are thermodynamically stabilised by calcium (e.g. trypsin or α-chymotrypsin), through deprivation of the enzyme-bound calcium (Lueβen et al., 1995; Ameye et al., 2001). It has also been demonstrated that poly(acrylates) are able to deplete extracellular Ca^{2+} whereby the paracellular permeability through the epithelium is increased by opening the tight junctions (Borchard et al., 1996). However, Ca^{2+}-poly(acrylates) exhibit different characteristics compared to Na^{+}-poly(acrylates). Complexation of calcium by poly(acrylic acid) derivates decreased the particle size of dispersed swollen particles of these polymers as calcium acts as cross-linking agent between polymer chains (Kriwet et al., 1996). Madsen and Peppas (1999) demonstrated that calcium decreased the swelling ratio of gels containing grafted copolymer networks of poly(metacrylic acid-g-ethylene glycol) significantly compared to the swelling ratio obtained in buffers containing NaCl. This phenomenon is pH-dependent: in acid media (below pH 4.4) monovalent nor divalent cations affect the swelling ratio as the protonated carboxylic acid groups are not available for sodium or calcium binding. At increasing pH, the acid groups are

ionised whereby sodium or calcium ions can interact with the polymer. Sodium ions (monovalent cations) can move freely in the poly(acrylate) network, whereas calcium ions (divalent cations) promote aggregation of polymer chains by intermolecular bridge formation (Horkay et al., 2001). The electrostatic repulsion between the negatively charged carboxylic acid groups is lower for calcium poly(acrylates), resulting in a decrease of the swelling force and consequently a much lower equilibrium swelling ratio (Madsen and Peppas, 1999).
This explained the decrease in t_{max} at higher $Ca(OH)_2$ content. At a ratio of 90/20 and 90/30 a weaker gel was obtained after dispersion in simulated nasal fluid compared to the formulation without $Ca(OH)_2$ (Table 4). The formulation at a ratio of 90/30 was even not able to form a homogeneous hydrated network, hence the viscosity and elasticity after dispersion in the nasal fluid could not be measured. This may result in a faster clearance of the formulations from the nasal cavity resulting in a decreased t_{max}.

From Table 4 it can also be observed that specifically the formulation based on (SD 25/75)/$Ca(OH)_2$ 90/10 exhibited a higher elasticity after dispersion in the nasal fluid compared to the reference formulation without $Ca(OH)_2$. Although this can provide an explanation for the enhanced absorption observed for the formulation, it does not explain the shorter t_{max}. The highest absorption observed for this formulation is probably due to an optimal balance between Na^+- and Ca^{2+}-carboxylate groups on the poly(acrylate) chains.

Table 4. Elasticity (G') and viscosity (G") of 10 % (w/w) dispersions into simulated nasal fluid.

Formulation	**G' (Pa)**	**G" (Pa)**
SD 25/75	1699 ± 31	142 ± 4
(SD 25/75)/$Ca(OH)_2$ 90/1	1673 ± 49	136 ± 3
(SD 25/75)/$Ca(OH)_2$ 90/10	1822 ± 26	152 ± 6
(SD 25/75)/$Ca(OH)_2$ 90/20	1412 ±176	113 ±15
(SD 25/75)/$Ca(OH)_2$ 90/30	-	-

Through the strong interaction between Ca^{2+} and the carboxylate groups, only a minimal concentration of free Ca^{2+}-ions will be present in the formulation. It is improbable that dissociated free Ca^{2+}-ions enhanced the paracellular absorption of insulin through the epithelium via the tight junctions. The integrity of the intercellular junctions can be modulated by divalent cations (e.g. Ca^{2+} and Mg^{2+}): a reduction of extracellular calcium as well as increased intracellular calcium levels can improve paracellular absorption by opening of the tight junctions (Bhat et al., 1993; Brown and Davis, 2002). Therefore, loading the nasal cavity with additional free Ca^{2+}-ions should rather reduce the nasal absorption of insulin. As for the formulations based on the ratio 90/1, 90/10 and 90/20 a higher C_{max} was observed in comparison with the reference formulation without $Ca(OH)_2$, it is demonstrated indirectly that Ca^{2+} is fixed on the carboxylate groups. On the other hand after dispersion of these formulations in nasal fluid, a progressive dissociation of Ca^{2+} from Ca^{2+}-carboxylate might be responsible for their shorter t_{max} due to the closing of the tight junctions in response to the dissociated Ca^{2+}-ions.

This hypothesis was also suggested in a preliminary study wherein the mechanism of absorption enhancement of the SD 25/75 and (SD 25/75)/$Ca(OH)_2$ 90/10 powders was investigated using TEER measurements. Assessment of the transepithelial electrical resistance (TEER) was used as direct method to estimate the opening of the tight junctions.
It was observed that hydrated SD 25/75 or (SD 25/75)/$Ca(OH)_2$ 90/10 particles had no effect on the TEER of the Caco-2 monolayers. In a second experiment, dry SD 25/75 or (SD 25/75)/$Ca(OH)_2$ 90/10 powder particles were spread on the apical side of the monolayers and after an incubation time of 2 or 5 min 500 µl calcium-free transport medium was added. After exposure of the epithelial cells to the dry powders a decrease in TEER was observed (Figure 3). This can be explained by the water absorbing capacity of the particles which dehydrated the epithelial cells causing a hydrostatic pressure within the paracellular space and opening of the tight junctions. A similar observation was made by Bjork et al. (1995) who found that only dry degradable starch microspheres were able to enhance the ^{3}H-mannitol transport across the Caco-2 cell monolayers.

After the incubation period the formulation (SD 25/75)/$Ca(OH)_2$ 90/10 induced a slightly higher decrease in TEER compared to SD 25/75. This suggested that the former had a higher water absorbing capacity. Evaluation of SNF uptake of both formulations demonstrated that (SD 25/75)/$Ca(OH)_2$ 90/10 had a slightly higher and faster absorption of SNF what correlated

with the TEER data (Figure 4). As the decrease in TEER is a measure for opening the tight junctions a slightly higher paracellular transport may be obtained for (SD 25/75)/$Ca(OH)_2$ 90/10, explaining the higher and faster absorption of insulin when this formulation was used as carrier.

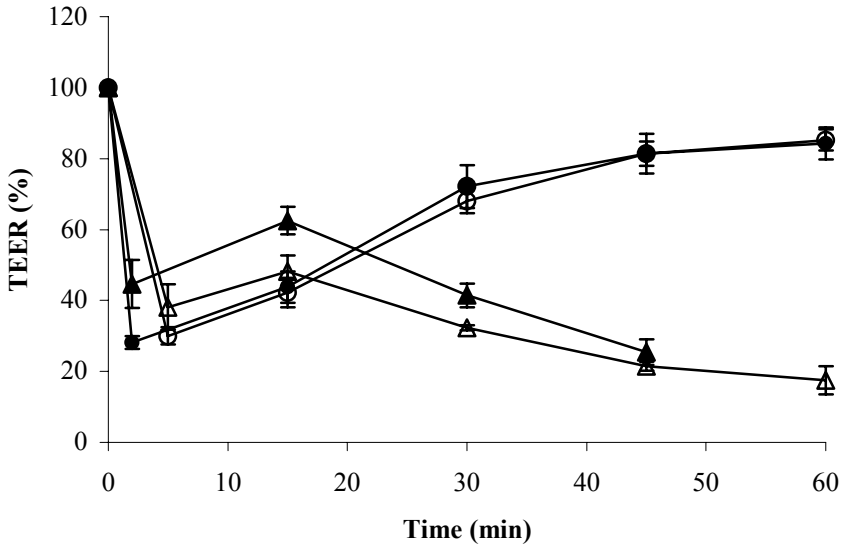


Figure 3. Influence of powder formulations SD 25/75 (▲) and (SD 25/75)/$Ca(OH)_2$ 90/10 (●) on the TEER values of Caco-2 cells after an incubation period of 2 (closed symbols) and 5 min (open symbols).

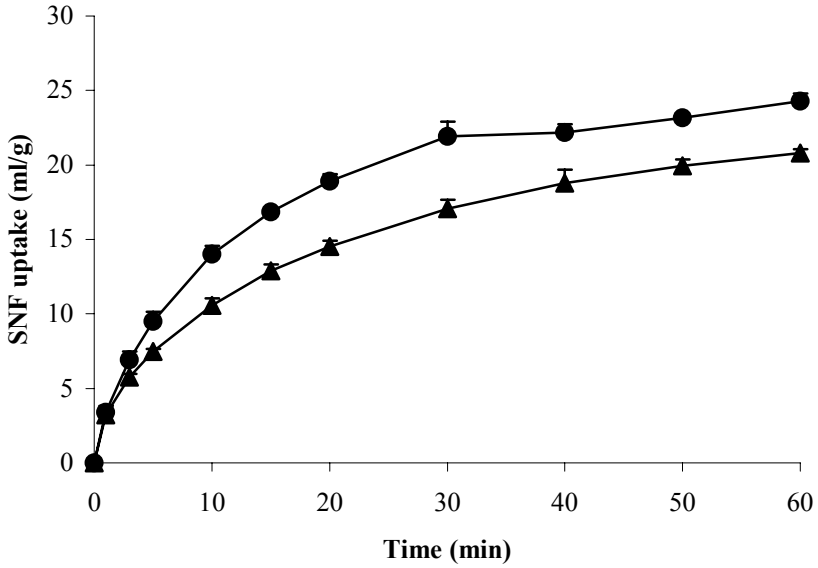


Figure 4. Simulated nasal fluid uptake by SD 25/75 (▲) and (SD 25/75)/$Ca(OH)_2$ 90/10 (●) powders (n = 2, mean ± SD).

Using transmission electron microscopy it was observed that 15 min after nasal administration of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 to rabbits the tight junctions were opened (Figure 5). However, no difference in the extent of opening between both formulations could be observed

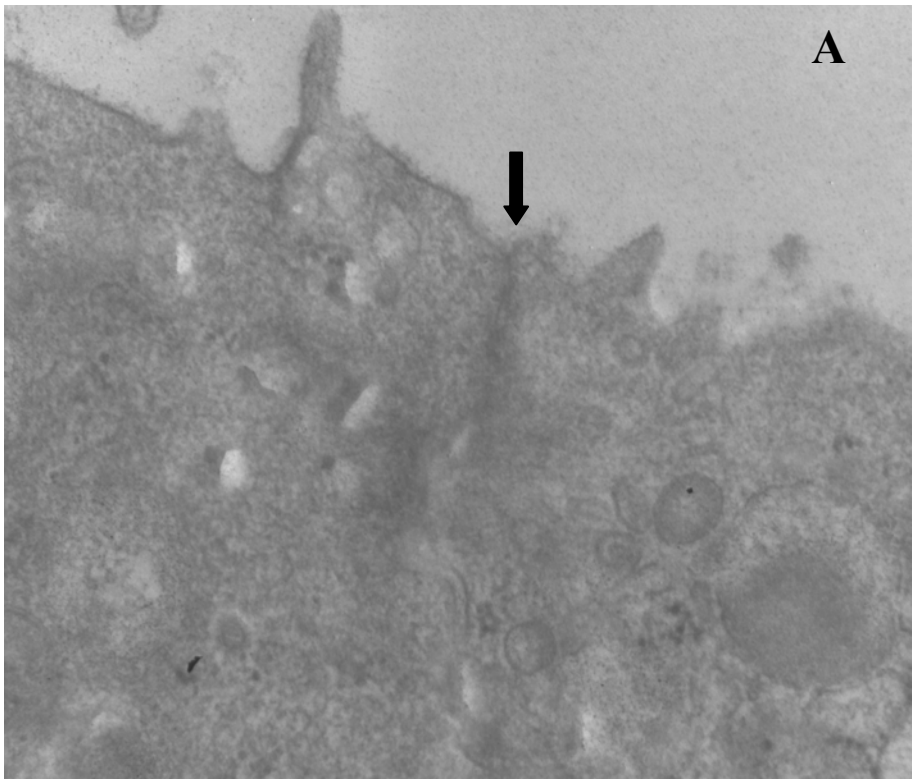
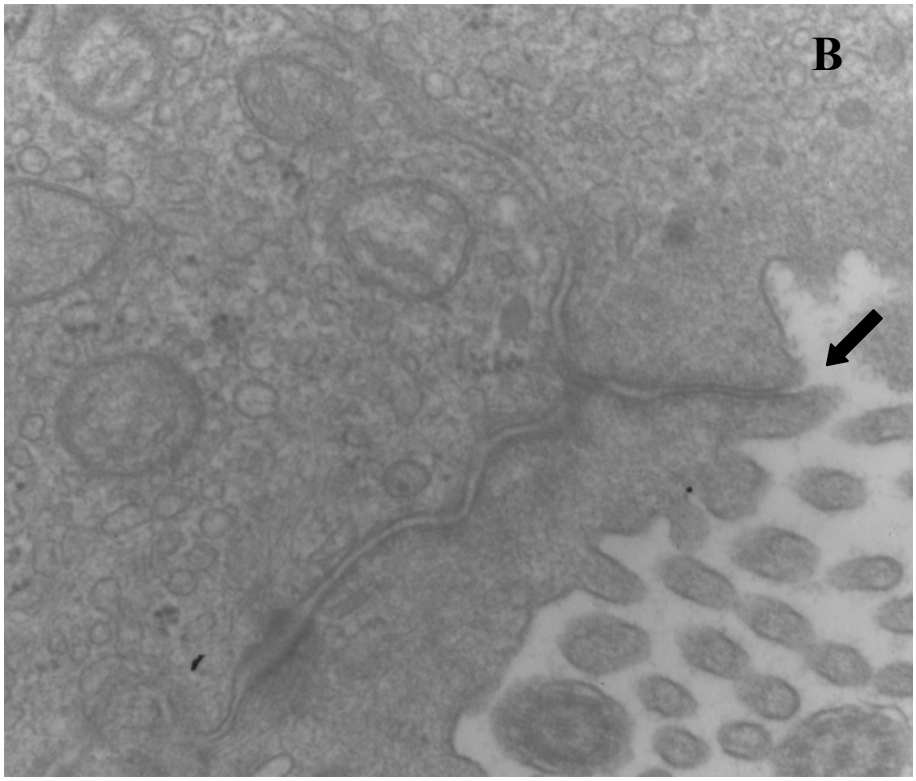


Figure 5. Transmission electron microscopy pictures of the closed tight junctions before nasal administration (A) (magnification 30000 x) and of the opened tight junctions 15 min after nasal administration of SD 25/75 (B) (magnification 30000 x) and (SD 25/75)/$CaCO_3$ 90/10 (C) (magnification 20000 x) to rabbits.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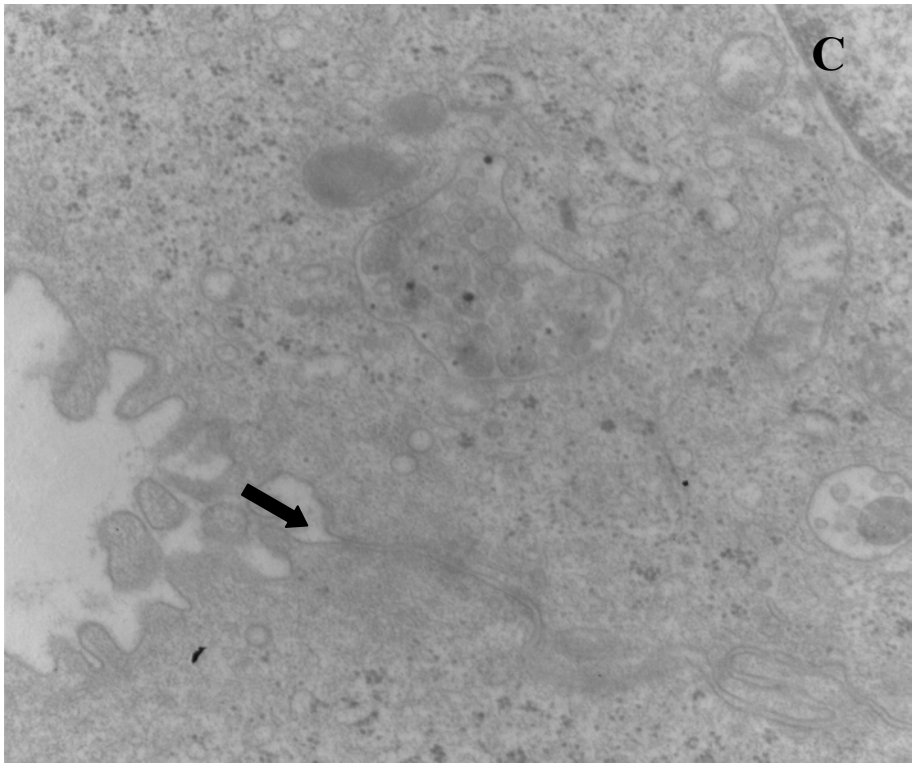
C

After addition of calcium-free transport medium the TEER was followed in function of time. Calcium-free transport medium was used to allow detection of a possible effect of dissociated calcium ions on membrane integrity. For the (SD 25/75)/$Ca(OH)_2$ 90/10 formulation a progressive increase in TEER was observed in function of the time (after 60 min the TEER values were ± 15 % lower than the initial values), while for the SD 25/75 powder formulation a further decrease in TEER was found (after 60 min the TEER values were ± 80 % lower than the initial values) (Figure 3). This suggested that calcium ions gradually dissociated from the Ca^{2+}-carboxylates. In Madin Darby canine kidney epithelial cells, a decreased transepithelial resistance due to decreased extracellular calcium levels was restored by calcium replacement (Balda et al., 1991). In primary pulmonary endothelial cultures, chelation of extracellular calcium increased the permeability of albumin, decreased the transepithelial resistance and caused retraction of adjacent cells. The normal barrier function was restored by repletion of calcium (Sasby and Sasby, 1986). Junctional resealing was also observed in native oesophageal epithelium when changing from calcium-free medium to calcium-containing solutions (Tobey et al., 2004). The increase in TEER observed for (SD 25/75)/$Ca(OH)_2$ 90/10 after hydration might be contributed to repletion of extracellular calcium via dissociated calcium ions, thus closing the tight junctions. This can explain the low t_{max} seen for the formulations containing Ca^{2+}-poly(acrylates). The further decrease in TEER after hydration of the SD 25/75 formulation could not be contributed to the calcium-free transport medium as medium compatibility with the Caco-2 monolayers has been demonstrated in preliminary tests. A further water absorption of the polymers in function of time might be a possible explanation. The prolonged decrease in TEER indicated that the tight junctions were opened for a longer period what may explain the higher t_{max} observed for this formulation.

As some problems were reported concerning quantitative transfer of the powder formulations to the Caco-2 cells and homogeneous spreading of the particles over their surface, the results of this TEER experiment have to be considered as preliminary and have to be confirmed in an additional experiment.

4.1.3.2 Insulin versus salmon calcitonin

The next experiment was performed to evaluate if a similar trend in bioavailability was observed when insulin (MW 5808 Da) was substituted by the smaller peptide salmon calcitonin (MW ± 3500 Da). Salmon calcitonin was used as it is more potent than human calcitonin in mammals, especially in man (Guttman, 1980).

In Figure 6 the salmon calcitonin concentration versus time profile after intravenous administration of 10 IU salmon calcitonin to rabbits is shown. The basal levels of calcitonin were determined in untreated rabbits and varied between 14 and 17 pg/ml (Figure 7). As these concentrations were not negligible in comparison with the concentrations obtained after nasal delivery of salmon calcitonin, the basal levels were taken into account for the calculation of the absolute bioavailability.

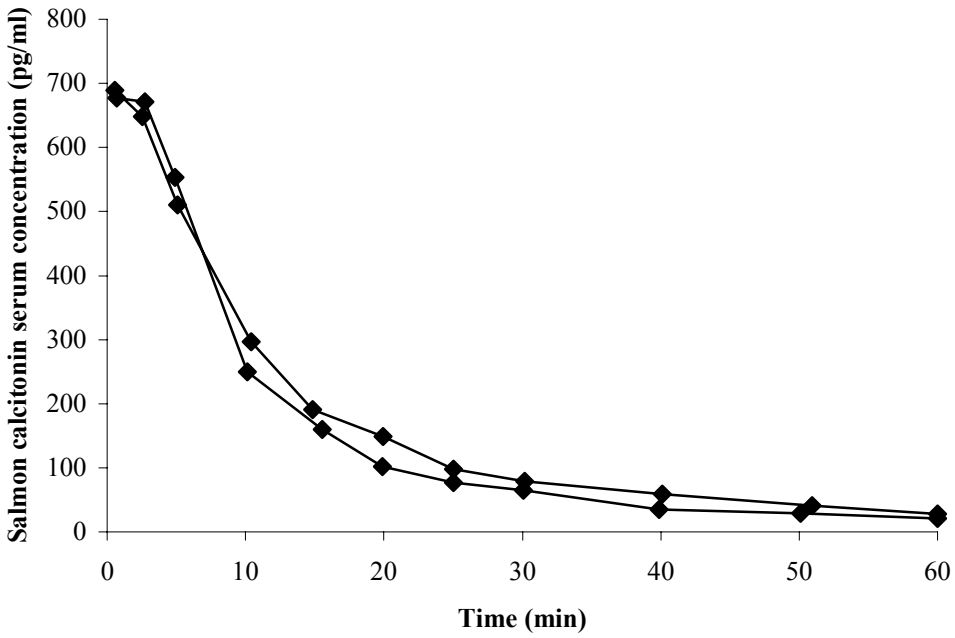

Figure 6. Salmon calcitonin serum concentration-time profiles after intravenous administration of 10 IU salmon calcitonin to rabbits (n = 2).

The salmon calcitonin serum concentration-time profiles obtained after nasal delivery of SD 25/75 and (SD 25/75)/$Ca(OH)_2$ 90/10 are given in Figure 7. It can be observed that

incorporation of salmon calcitonin into the carriers resulted in a similar trend as for insulin. Although the differences were not significant ($P > 0.05$), the absolute bioavailability and C_{max} were higher while t_{max} was lower when SD (25/75)/$Ca(OH)_2$ 90/10 was used as carrier (Table 5).

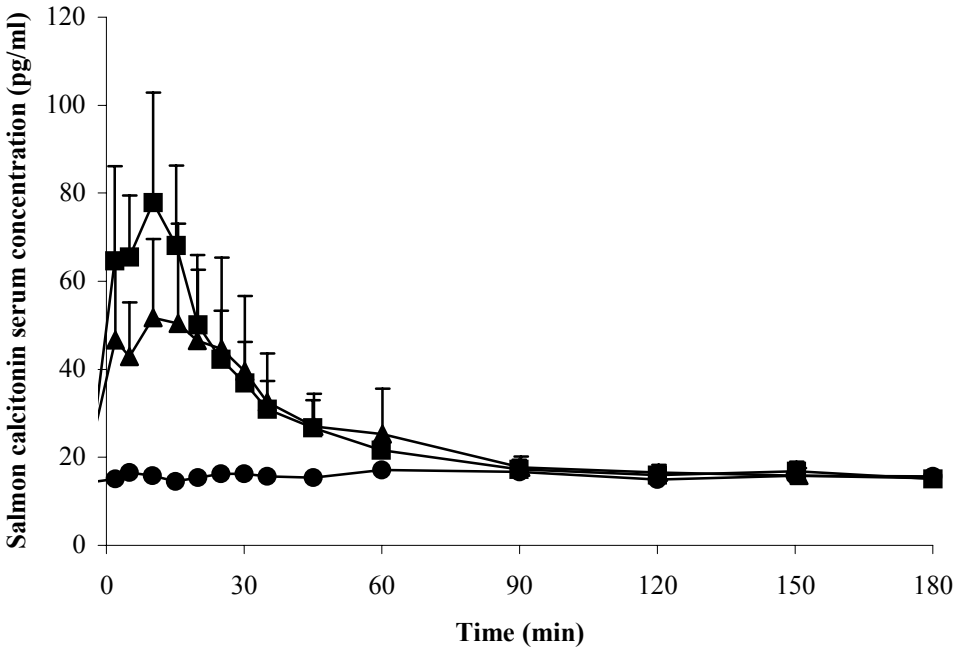

Figure 7. Basal serum levels (●) and serum concentration-time profiles of salmon calcitonin after nasal delivery to rabbits of powders (2 IU salmon calcitonin/mg) based on SD 25/75 with $CaCO_3$ (■) and without $CaCO_3$ (▲).

Table 5. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) of salmon calcitonin formulated in nasal powder formulations (2 IU salmon calcitonin/mg) containing SD 25/75 or (SD 25/75)/$Ca(OH)_2$ 90/10.

Formulation	BA (%)	C_{max} (pg/ml)	t_{max} (min)	n
SD 25/75	5.8 ± 4.1	56.5 ± 18.3	15.8 ± 4.1	4
(SD 25/75)/$Ca(OH)_2$ 90/10	7.6 ± 2.7	81.3 ± 23.2	10.8 ± 3.2	4

Although the nasal bioavailability of hydrophilic molecules generally decreased with increasing molecular weight (Pontiroli and Pozza, 1990; Behl et al., 1998), the nasal bioavailability of salmon calcitonin was dramatically lower compared to equivalent formulations with insulin. This may be due to the different enzyme specificity as well as to the concentration and activity of both proteins (Hayakawa et al., 1989; Gizurarson and Bechgaard, 1991; Ishikawa et al. 2001).

4.1.3.3 Spray-drying versus physical mixing

The importance of using a spray-dried mixture of Amioca® starch and Carbopol® is emphasised in Figure 8 and Table 6. Nasal delivery of powders based on a physical mixture of Amioca® starch and Carbopol® 974P resulted in a significant ($P < 0.001$) lower absorption of insulin. C_{max} was significantly ($P = 0.001$) lower, while a similar ($P > 0.05$) t_{max} was obtained.

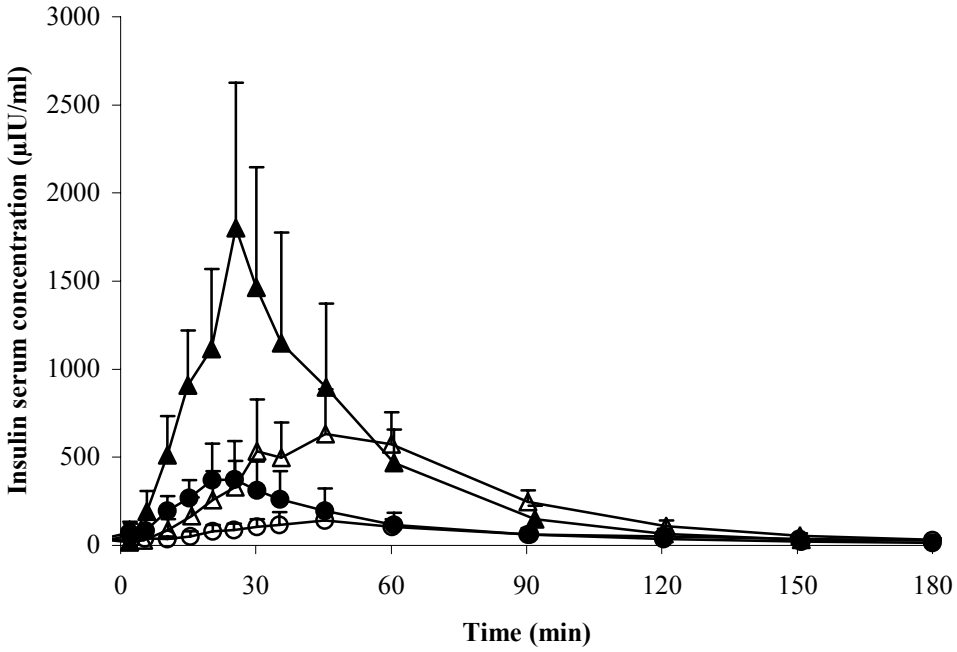

Figure 8. Insulin serum concentration-time profiles after nasal delivery to rabbits of powders (1 IU insulin/mg) based on SD 25/75 (▲) and PM 25/75 (●) with $Ca(OH)_2$ (closed symbols) and without $Ca(OH)_2$ (open symbols).

Table 6. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) of insulin after nasal delivery to rabbits of powders (1 IU insulin/mg) based on SD 25/75 and PM 25/75 with and without the addition of $Ca(OH)_2$.

Formulation	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
[a] SD 25/75	19.2 ± 5.3 ***	681 ± 247 ***	50.9 ± 7.4	8
(SD 25/75)/$Ca(OH)_2$ 90/10	29.0 ± 11.4	1813 ± 828	27.3 ± 3.4	8
[b] PM 25/75	4.9 ± 1.6	228 ± 178	47.8 ± 8.3	8
[c] (PM 25/75)/$Ca(OH)_2$ 90/10	8.2 ± 4.3	385 ± 217	24.1 ± 3.4 ***	8

Set[a] was compared to set[b] using Contrast Analysis (Significantly higher: *** = $P \leq 0.001$).

Set[b] was compared to set[c] using Contrast Analysis (Significantly lower: *** = $P \leq 0.001$).

The reduced absorption of the formulation based on the physical mixture can be attributed to the slower and lower hydration of the formulation compared to the equivalent spray-dried mixture (Figure 9). The uptake of insulin incorporated in PM 25/75 started 15 min after administration, while the onset of insulin absorption using the carrier SD 25/75 was within 5 min after nasal delivery.

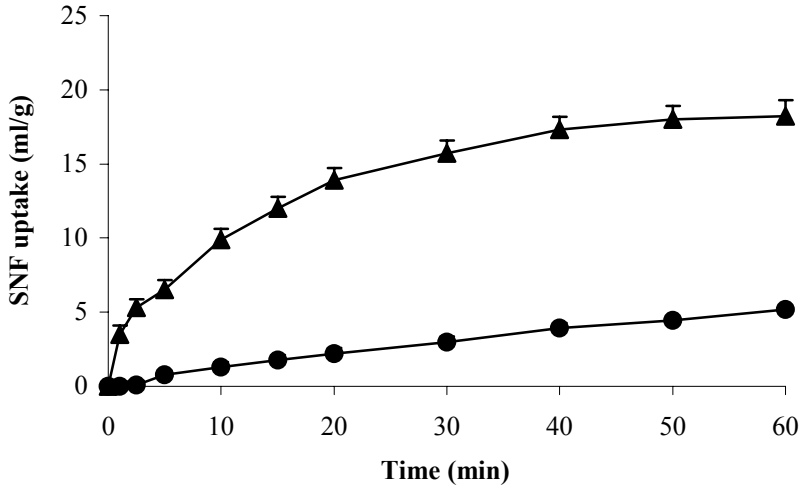

Figure 9. Simulated nasal fluid uptake by SD 25/75 (▲) and PM 75/25 (●) powder (n = 2, mean ± SD).

Creating a Ca^{2+}-poly(acrylate) starting from a physical mixture of PM 25/75 and $Ca(OH)_2$ in a ratio 90/10, the absorption of insulin was promoted compared to the equivalent formulation without $Ca(OH)_2$: the absolute nasal bioavailability and C_{max} were increased ($P > 0.05$) and t_{max} was decreased ($P < 0.001$) (Figure 8, Table 6).

4.1.4 Conclusions

Freeze-drying neutralised (using NaOH) aqueous dispersions containing Amioca® starch, Carbopol® 974P and $Ca(OH)_2$ (or $CaCO_3$) yielded powders containing a mixture of sodium and calcium carboxylate. There was an optimal balance between sodium and calcium carboxylate to enhance the absorption of insulin which was obtained for a formulation containing 10 % $Ca(OH)_2$ or $CaCO_3$. After nasal peptide delivery using these formulations a higher C_{max} but lower t_{max} was observed in comparison with the equivalent formulations without $Ca(OH)_2$. The mechanism of absorption enhancement was possibly due to the higher water absorbing capacity of the Ca-containing powder and to its higher elasticity after dispersion into the nasal fluid. The decrease in t_{max} when the calcium concentration in the formulation increased was possibly due to the progressive dissociation of calcium from the Ca^{2+}-carboxylate after hydration of the powder, inducing a closure of the tight junctions.
The importance of using a spray-dried starch/Carbopol® mixtures instead of a physical mixture was also demonstrated as this contributed to the water absorbing capacity.

4.1.5 References

Ameye D., Voorspoels J., P. Foreman P., Tsai J., Richardson P., Geresh S., Remon J.P., Trypsin inhibition, calcium and zinc ion binding of starch-g-poly(acrylic acid) copolymers and starch/poly (acrylic acid) mixtures for peroral peptide drug delivery. J. Control. Rel. 75 (2001) 357-364.

Balda M.S., Gonzalez-Mariscal L., Contreras R.G. Macias-Silva M., Torres-Marquez M.E., Garcia Sainz J.A., Cereijido M., Assembly and sealing of tight junctions: possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. J. Membr. Biol. 122 (1991) 193-202.

Behl C.R., Pimplaskar H.K., Sileno A.P., Xia W.J., Gries W.J., deMeireles J.C., Romeo V.D., Optimization of systemic nasal drug delivery with pharmaceutical excipients. Adv. Drug Del. Rev. 29 (1998) 117-133.

Bhat M., Toledovelasquez D., Wang L., Malanga C.J., Ma J.K.H., Rojanasakul Y., Regulation of tight junction permeability by calcium mediators and cell cytoskeleton in rabbit tracheal epithelium. Pharm. Res. 10 (1993) 991-997.

Björk E., Isaksson U., Edman P., Artursson P., Starch microspheres induce pulsatile delivery of drugs and peptides across the epithelial barrier by reversible separation of the tight junctions. J. Drug Target. 2 (1995) 501-507.

Borchard G., Lueβen H.L., Deboer A.G., Verhoef J.C., Lehr C.M., Junginger H.E., The potential of mucoadhesive polymers in enhancing intestinal peptide drug absorption. 3. Effects of chitosan-glutamate and carbomer on epithelial tight junctions in vitro. J. Control. Rel. 39 (1996) 131-138.

Brown R.C., Davis T.P., Calcium modulation of adherens and tight junction function - a potential mechanism for blood-brain barrier disruption after stroke. Stroke 33 (2002) 1706-1711.

Callens C., Remon J.P., Evaluation of starch–maltodextrin–Carbopol® 974 P mixtures for the nasal delivery of insulin in rabbits. J. Contr. Rel. 66 (2000) 215-220.

Callens C., Pringels E., Remon J.P., Influence of multiple nasal administrations of bioadhesive powders on the insulin bioavailability. Int. J. Pharm. 250 (2003) 415-422.

Cilurzo F., Selmin F., Minghetti P., Montanari L., The effects of bivalent inorganic salts on the mucoadhesive performance of a polymethylmethacrylate sodium salt. Int. J. Pharm. 301 (2005) 62-70.

Crisp S., Pringuer M.A., Wardleworth D., Wilson A.D., Reactions in glass ionomer cements: II. An infrared spectroscopic study. J. Dent. Res. 53 (1974) 1414-1419.

Deb S., Nicholson J.W., The effect of strontium oxide in glassionomer cements. J. Mater. Sci. Mater. Med. 10 (1999) 471-474.

Geresh S., Gdalevsky G.Y., Gilboa I., Voorspoels J., Remon J.P., Kost J., Bioadhesive grafted starch copolymers as platforms for peroral drug delivery: a study of theophylline release. J. Contr. Rel. 94 (2004) 391-399.

Gizurarson S., Bechgaard E., In vitro study of nasal enzyme activity towards insulin. Chem. Pharm. Bull. 39 (1991) 2155-2157.

Guttman S., Chemistry and structure-activity relationship of natural and synthetic calcitonins. In: Pecile A. (Ed.), Calcitonin 1980: Chemistry, physiology, pharmacology and clinical aspects. Excerpta Medica, Amsterdam, 1980, 11-24.

Hayakawa E., Yamamoto A., Shoji Y., Lee V.H.L., Effect of sodium glycolate and polyoxyethylene-9-lauryl ether on the hydrolysis of varying concentrations of insulin in the nasal homogenates of the albino rabbit. Life Sci. 45 (1989) 167-174.

Horkay F., Tasaki I., Basser P.J., Effect of monovalent-divalent cation exchange on the swelling of poly(acrylate) hydrogels in physiological salt solutions. Biomacromolecules 2 (2001) 195-199.

Ishikawa F., Katsura M., Tamai I., Tsuji A., Improved nasal bioavailability of elcatonin by insoluble powder formulation. Int. J. Pharm. 224 (2001) 105-114.

Kriwet B., Kissel T., Interactions between bioadhesive poly(acrylic acid) and calcium ions. Int. J. Pharm. 127 (1996) 135-145.

Lee C.H., Chien Y.W., Development and evaluation of mucoadhesive drug delivery systems for dual-controlled delivery of nonoxynol-9. Int. J. Pharm. 39 (1996) 93-103.

Lueβen H.L., Verhoef J.C., Borchard G., Lehr C.M., Deboer A.G., Junginger H.E., Mucoadhesive polymers in peroral peptide drug-delivery. 2. Carbomer and polycarbophil are potent inhibitors of the intestinal proteolytic-enzyme trypsin. Pharm. Res. 12 (1995) 1293-1298.

Madsen F., Peppas N.A., Complexation graft copolymer networks: Swelling properties, calcium binding and proteolytic enzyme inhibition. Biomaterials 20 (1999) 1701-1708.

Melon J., Contribution à l'étude de l'activité sécrétoire de la muqueuse nasale. Caractères de perméabilité de l'épithélium, composition chimique du mucus, le mécanisme de sa formation dans les conditions normales et au cours des hypersécrétions paroxystiques. Acta Oto-Rhino-Laryngol. Belg. 22 (1968).

Nicholson J.W., Brookman P.J., Lacy O.M., Sayers G.S., Wilson A.D., A study of the nature and formation of zinc polyacrylate cement using Fourier transform infrared spectroscopy. J. Biomed. Mater. Res. 22 (1988) 623-631.

Pontiroli A.E., Pozza G., Intranasal administration of peptide hormones: factors affecting transmucosal absorption. Diabetic Med. 7 (1990) 770-774.

Sasby D.M., Sasby S.S., Effects of calcium on transendothelial albumin transfer and electrical resistance. J. Appl. Physiol. 60 (1986) 71-79.

Sørensen A., Animal models for buccal and nasal delivery studies. In: Duchêne D. (Ed.), Buccal and nasal administration as an alternative to parenteral administration. European Symposium, Paris, 10-11 Dec, Editions de Santé (1991) 162-173.

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Tobey N.A., Argote C.M., Hosseini S.S., Orlando R.C., Calcium-switch technique and junctional permeability in native rabbit esophageal epithelium. Am. J. Physiol. Gastrointest. Liver Physiol. 286 (2004) 1042-1049.

Chapter 4.2 Mucosal irritation testing on rabbits and slugs

4.2.1 Introduction

A very important issue in the development of nasal formulations is the irritating potential of the active ingredient as well as the non-active components. Formulations for protein and peptide delivery are mostly indicated for chronic therapies whereby their safety is as essential as their effectiveness. For nasal delivery four areas of toxicity can be distinguished: local irritation of the mucosa, effect on mucociliary clearance, epithelial damage and rate of recovery of the damaged epithelium (Agu et al., 2002). Different in vitro and in vivo methods have been developed to assess the toxicological effects of nasal formulations. An erythrocyte model has been used to demonstrate the membrane activity of different absorption enhancers such as bile salts, surfactants (Longenecker et al., 1987) and fatty acids (Mishima et al., 1987). Histopathological examination of the nasal mucosa gives a qualitative assessment of morphological damage caused by the formulation (Merkus et al., 1993). As mucociliary clearance is a local defence mechanism protecting the respiratory tract from invading microorganisms, a nasal formulation should not adversely affect the normal mucociliary clearance mechanism. Agu et al. (1999) used human nasal epithelial cell cultures to predict the nasal cilio-toxicity of pharmaceutical compounds in humans by measuring the ciliary beat frequency. The mucociliary clearance can also be followed in vivo by gamma scintigraphy or using a saccharin clearance test (Schipper et al., 1991).

The present study was performed to evaluate if the enhanced nasal bioavailability of insulin after administration of a powder formulation containing (SD 25/75)/$CaCO_3$ 90/10 (cfr. Chapter 4.1) is due to mucosal damage. In literature it has been reported that enhanced bioavailability is frequently associated with a disruption of the integrity of the mucosal epithelium.

The possible toxicological effects after multiple administration of the powders were evaluated using a non-invasive in vivo method in rabbits (Callens et al., 2001). After administration of the formulations the nasal cavity was washed with phosphate buffered saline to determine the release of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP), which are biochemical markers for membrane damage.

This method was compared with the Slug Mucosal Irritation (SMI) assay developed by Adriaens and Remon (1999). The use of invertebrates as model organism for screening toxicological effects of chemicals on mucosal surfaces is preferred based on ethical and financial considerations. The hermaphroditic slug *Arion Lusitanicus* was selected as the body wall of the slug is covered with a single-layered epithelium containing ciliated cells, cells with microvilli and mucus-producing cells. Mucus secretion is essential for locomotion of the slug and protects its body wall against mechanical and chemical damage (South, 1992). The amount of mucus produced by the slugs during repeated contact with a chemical substance is a measure for irritation, while tissue damage can be estimated from the release of proteins and enzymes from the body wall (Adriaens and Remon, 1999, 2002; Callens et al., 2001; Ceulemans et al., 2001; Adriaens et al., 2003; Dhondt et al., 2004, 2005; Weyenberg et al., 2004).

In the past a similar comparison showed a good correlation between the non-invasive in vivo method in rabbits and the SMI assay (Callens, 2002). A formulation containing Drum Dried Waxy Maize starch/Carbopol® 974P 90/10 (DDWM/C 974P 90/10) was administered nasally to rabbits during 28 consecutive days. Histological examination of the nasal epithelium at the end of the experiment revealed only a slight increase in the number of granulocytes. In the SMI test the same formulation was classified mildly irritating without causing membrane damage. For the negative control (untreated rabbits or slugs) an intact membrane in the nasal cavity of the rabbits was found after 28 days, which was in agreement with the results of the SMI assay where no irritation or membrane damage was seen. The positive control (DDWM/benzalkonium chloride 95/5), on the other hand, resulted in severe irritation and membrane damage in rabbits and slugs. Microscopic examination of the nasal epithelium of rabbits after daily treatment during only 5 days with the positive control showed a squamous epithelium with many granulocytes and ulcerations. Contact of the positive control with the foot mucosa of slugs induced severe irritation and irreversible damage of the mucosal tissue.

The present study describes the optimisation of the SMI procedure for the evaluation of viscosity-enhancing and/or bioadhesive polymers. Upon contact between dry powders containing viscosity-enhancing and/or bioadhesive polymers and the body wall of slugs, the powder absorbs water from the mucosal epithelium whereby a polymer film is formed over the foot mucosa of the slug. After a contact period of 30 min with the powder, the slugs are

transferred to a Petri dish containing phosphate buffered saline (PBS) solution. The PBS samples are analysed for the presence of proteins, LDH and ALP released from the body wall to evaluate the membrane damage. When the polymer film on the foot mucosa is not completely removed, it may form a barrier to the release of proteins and enzymes from the mucosa into the PBS solution, possibly underestimating the toxicological effects induced by the formulation. As a consequence the SMI procedure was modified to ensure complete removal of the polymer film from the foot mucosa of the slug. This was done by washing the foot mucosa with 2 ml PBS and cautiously drying with absorbent paper or by cleaning the foot mucosa with paper moistened with PBS before the slugs were transferred to the Petri dish containing PBS. The effect of these modifications on the protein and enzyme release and the prediction of the mucosal tolerance of the powders were evaluated.

4.2.2 Materials and methods

4.2.2.1 Materials

Phosphate buffered saline (PBS, pH 7.4) and sodium lauryl sulphate (SLS) were obtained from Sigma Diagnostica (Bornem, Belgium). The spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75, w/w) (SD 25/75) (batch number: 10851: 141-1) was prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Drum Dried Waxy Maize starch (DDWM) was obtained from Eridania-Béghin Say-Cerestar (Vilvoorde, Belgium). Calcium carbonate ($CaCO_3$) (density: 0.30 g/ml) was purchased from Federa (Brussels, Belgium). All other chemicals were of analytical grade.

4.2.2.2 Preparation of powder formulations

1.0 g SD 25/75 or 1.0 g of a physical mixture of SD 25/75 and $CaCO_3$ (ratio: 90/10, w/w) was dispersed in 40.0 ml distilled water and neutralised with 2.0 M NaOH to pH 7.4. Water was removed from the formulation by freeze-drying: the dispersion was frozen to 228 K within 175 min at 1000 mbar. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature

(283 K) and reduced pressure (0.1 - 0.2 mbar) for 7 h. After freeze-drying the powders were sieved (63 µm) at low relative humidity (20 %) and ambient temperature. The fractions below 63 µm were stored in a desiccator at 4 - 8 °C until use.

4.2.2.3 Irritation test on rabbits

4.2.2.3.1 Experimental procedure

During 5 consecutive days 10.0 mg of the nasal powders SD 25/75 or (SD 25/75)/$CaCO_3$ 90/10 was sprayed into each nostril of New Zealand white rabbits (n = 6, 3.0 ± 0.5 kg). The powders were administered using polyethylene tubes (Medisize, Hillegom, The Netherlands) connected to a syringe containing 1 ml compressed air (± 2.5 bar). This device was based on a system developed by Sørensen (1991).
1 and 23 h after administration each nostril was washed with 600 µl PBS (pH 7.4) using a micropipette to quantify the amount of proteins, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) released from the mucosa into the nasal cavity. During these manipulations the rabbits were in supine position, their back lifted to an angle of 45° and their head was kept horizontally to prevent drainage of the PBS into the nasopharynx. After 5 s PBS was collected in a test tube by returning the rabbits to their normal upright position. Release of the nasal epithelial cytosolic enzyme LDH served as an indicator for leakages caused by membrane damage or cell lysis, while the release of membrane-associated ALP gave an indication of membrane damage (Krishnamoorthy et al., 1995).

4.2.2.3.2 Analytical procedures

4.2.2.3.2.1 Determination of protein concentration

The total protein concentration in the PBS samples was determined with a NanoOrange® protein quantitation kit (Molecular Probes, Leiden, The Netherlands) (Harvey et al., 2001). The NanoOrange® reagent allowed accurate detection of proteins in solutions at concentrations between 10 ng and 10 µg/ml. Bovine serum albumin was used as a standard. The fluorescence measurements were carried out on a fluorometer (Wallac 1420 multilabel

counter, PerkinElmer, Turku, Finland) using excitation/emission wavelengths of 485/590 nm. The protein concentration was expressed as µg/ml.

4.2.2.3.2.2 Determination of LDH and ALP activity

The lactate dehydrogenase activity (LDH, EC 1.1.1.27) and alkaline phosphatase activity (ALP, EC 3.1.3.1) were measured with an enzyme kit (LDH/HBDH 2.8 and ALP 6, ABX diagnostics, Montpellier, France). The enzyme activity measurements were conducted on a Cobas Plus analyser (ABX, Brussels, Belgium) at 37°C. The enzyme activity was expressed as U/l.

4.2.2.3.3 Statistical analysis

Per sampling point the mean protein concentration, LDH activity and ALP activity of both nostrils were calculated.
Statistical analysis of the data was performed using SPSS version 11.0. To evaluate the effect of multiple powder administration on protein, LDH and ALP release from the nasal mucosa of rabbits a Repeated Measures Univariate Variance-Analysis was performed. The sphericity of the data was tested using the Mauchly's test. If sphericity of the data was not fulfilled, a correction was made with the Huyn-Feldt test. The homogeneity of the variances was tested using the Levene's test. To compare the nasal washings between the different powders and within one powder formulation a Bonferroni test was used with $P < 0.05$ as significance level.

4.2.2.4 Slug Mucosal Irritation test

4.2.2.4.1 Experimental procedure of Slug Mucosal Irritation test

The mucosal irritation test according to the methodology described by Adriaens and Remon (1999) was modified for viscosity-enhancing and/or bioadhesive powders (Callens et al., 2001) and will be described briefly. Slugs weighing between 2.5 and 4.5 g were used. The experiment contained five negative control slugs (placed on 20 mg DDWM), five positive control slugs (placed on 20 mg DDWM/sodium lauryl sulphate

(ratio: 80/20, w/w) (DDWM/SLS)) and per test formulation five slugs (placed on 20 mg SD 25/75 or (SD 25/75)/$CaCO_3$ 90/10). The slugs were placed daily on the powder formulations during 30 min for 5 successive days. The amount of mucus produced during each contact period was determined. After the 30-min contact period, the slugs were transferred to a Petri dish containing 1 ml PBS (pH 7.4). After 1 h PBS was collected with a micropipette and the slugs were placed for another 1 h on a new Petri dish containing 1 ml PBS after which PBS was again collected. PBS samples were analysed for proteins, LDH and ALP released from the body wall. After sampling the slugs were placed on a membrane filter (cellulose acetate 0.45 µm, Sartorius, Goettingen, Germany) moistened with 2 ml PBS until the next contact period (Experiment I). A schematic overview of the procedure is given in Figure 1.

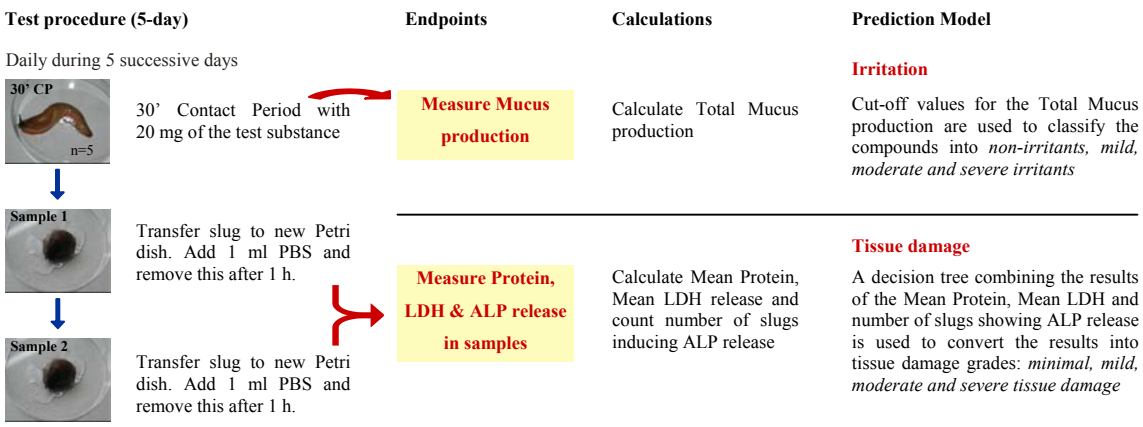

Figure 1. Overview of the Slug Mucosal Irritation test procedure, this procedure was repeated during 5 successive days.

Two modifications of the procedure as described above were examined. For those experiments the same procedure was used, however before transferring the slugs to a Petri dish after the 30-min contact period with the formulation, the foot mucosa of the slugs was washed with 2 ml PBS and dried with absorbent paper (Experiment II) or the foot mucosa was cleaned with paper moistened with PBS (Experiment III).

4.2.2.4.2 Determination of endpoints

4.2.2.4.2.1 Mucus production

The amount of mucus produced during each contact period was measured by weighing the Petri dishes with the test item before and after each 30-min contact period. The mucus production was expressed as % of the body weight. The slugs were weighed before and after each 30-min contact period.

4.2.2.4.2.2 Determination of protein concentration

The protein concentration was determined as described in 4.2.2.3.2.1, but the protein concentration was expressed as µg/ml per g body weight.

4.2.2.4.2.3 Determination of LDH and ALP activity

The LDH and ALP activity were determined as described in 4.2.2.3.2.2, but the enzyme activity was expressed as U/l per g body weight.

4.2.2.4.3 Interpretation of the results

The irritation potency and tissue damaging effect of the test substances were estimated using a classification prediction model based on the endpoints of the SMI assay. The irritation potency was estimated by the total mucus production (4 classes: non-irritant, mild, moderate and severe irritant). The cut-off values for solids were used for classification. The total mucus production was calculated by averaging the mucus production of all slugs measured during each 30-min contact period.
The tissue damage was predicted by the mean protein and LDH release and by the number of slugs showing ALP release (4 classes: minimal, mild, moderate and severe membrane damage). The overall mean protein release was calculated from the samples collected between day 2 until day 5 for each slug. For the LDH release the overall mean of the samples was calculated from all data points collected for each slug. The classification prediction model is depicted in Figure 2.

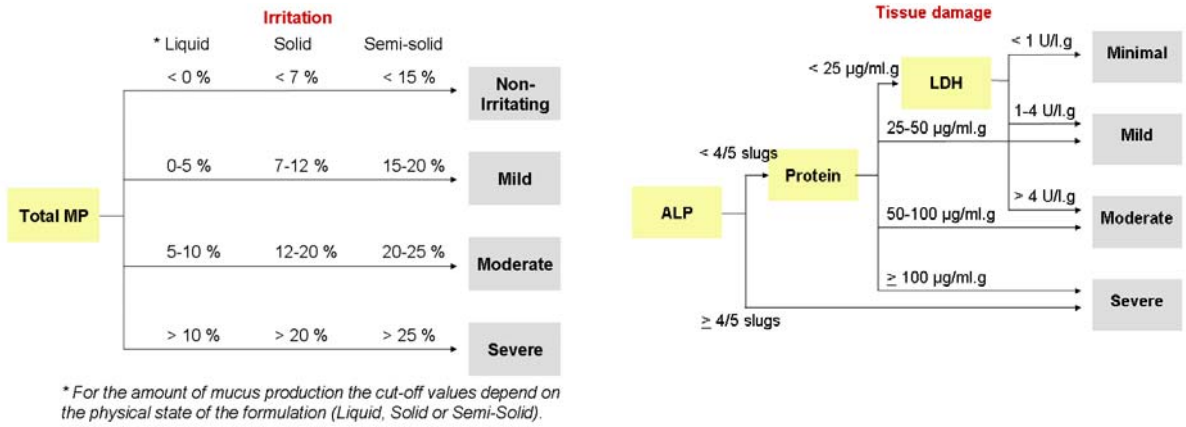

Figure 2. The Slug Mucosal Irritation prediction model that distinguishes between irritation potency and tissue damage.

4.2.2.4.4 Acceptance criteria

Before the results of the conventional set-up of the SMI assay (Exp. I) are considered valid, the following criteria had to be met:

- the negative control (DDWM) should be classified as non-irritant (total mucus production < 7 %) and should induce minimal tissue damage (mean protein release < 25 µg/ml.g, mean LDH release < 1 U/l.g and no ALP release)
- the positive control (DDWM/SLS 80/20) should be classified as a severe irritant (total mucus production > 20%) and should result in severe tissue damage (ALP release in 4 of the 5 slugs or mean protein release > 100 µg/ml.g)

4.2.3 Results

4.2.3.1 Nasal irritation study on rabbits

The aim of this study was to compare the mucosal toxicity of the nasal powder formulation SD 25/75 with (SD 25/75)/$CaCO_3$ 90/10 after multiple administration to rabbits. Mucosal damage was monitored by determining the release of proteins and the two marker enzymes,

LDH and ALP, in the washings of the nasal cavity as described in the method developed by Callens et al. (2001).

4.2.3.1.1 Preliminairy study

In a preliminary study the protein concentration and the activity of LDH and ALP were determined in nasal washing collected 1 h before and 1 and 23 h after single nasal administration of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 to rabbits. Washing 1 h after administration was done to investigate if an increased protein, LDH and ALP release occurred which is an indication of membrane damage. Washing of the nasal cavity 23 h after administration was performed to evaluate if the release of proteins and marker enzymes had returned to their basal level. This allowed to estimate the reversibility of membrane damage induced by the powders. The protein concentration and enzyme activity measured before administration were used as basal levels.

This preliminary test was performed to evaluate the reproducibility of the method reported by Callens et al. (2001). The results are summarised in Table 1.

Table 1. Protein concentration and LDH and ALP activity found in nasal washings performed 1 h before and 1 and 23 h after a single nasal administration of the powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 to rabbits.

	1 h before	1 h after	23 h after	n
LDH (U/l)				
SD 25/75	170 ± 172 [a]	750 ± 347 [b]	284 ± 107 [a]	4
(SD 25/75)/$CaCO_3$ 90/10	253 ± 295 [a]	738 ± 413 [b]	379 ± 408 [a]	8
ALP (U/l)				
SD 25/75	130 ± 166 [a]	236 ± 177 [a]	184 ± 70 [a]	4
(SD 25/75)/$CaCO_3$ 90/10	90 ± 61 [a]	146 ± 66 [a]	152 ± 87 [a]	8
PROTEINS (µg/ml)				
SD 25/75	1355 ± 842 [a]	2308 ± 792 [a]	1687 ± 554 [a]	4
(SD 25/75)/$CaCO_3$ 90/10	1562 ± 597 [a]	1808 ± 563 [a]	1595 ± 845 [a]	8

Data are presented as mean ± SD.

Within each formulation the same subscripts are not significantly different from each other (P > 0.05, Bonferroni test).

For both formulations an increase in protein concentration and enzyme activity was measured in the PBS samples 1 h after administration. Only for LDH the increase was significant ($P < 0.05$). One hour after administration the differences in protein, LDH and ALP release between both treatments were not significant ($P > 0.05$), however the values for SD 25/75 were higher than for (SD 25/75)/$CaCO_3$ 90/10.

After 23 h the levels of proteins and enzymes in the nasal washings had decreased, although they remained slightly higher, but not significantly ($P > 0.05$), than the levels measured before administration. The decrease in protein and enzyme release 23 h after administration indicated that the effects of the powders on the nasal mucosa were reversible after single administration.

Callens (2002) administered the SD 25/75 powder daily to rabbits during 28 days. Every 3-4 days the nasal cavity was washed before and after administration to determine the protein concentration and enzyme activity. The results were compared to untreated rabbits (negative control group) and to rabbits treated with DDWM/benzalkonium chloride during 12 consecutive days (positive control group). The results of these experiments are shown in Table 2.

Callens (2002) reported a similar trend after nasal delivery of SD 25/75 during 28 days as in the preliminary experiment after single administration: increased levels of proteins, LDH and ALP were found immediately after administration and these levels had decreased after 23 h. The protein concentration and enzyme activity after administration were clearly higher and lower compared to the negative and positive control, respectively. Furthermore, it was noted that the protein concentration and enzyme activity 1 h after administration of SD 25/75 was independent of the number of administrations, suggesting that the membrane damage is reversible. Moreover, in our preliminary experiment the levels of proteins, LDH and ALP 1 h after a single administration of SD 25/75 were in the same range of the values of the mean protein concentration and mean enzyme activity measured during multiple administration. This demonstrated that the non-invasive washing method described by Callens et al. (2001) is reproducible, therefore the negative and positive control experiments were not repeated based on ethical considerations and the data generated by Callens et al. (2001) will be used as reference values in this study.

Table 2. Mean protein concentration and mean LDH and ALP activity measured in nasal washings of untreated rabbits (negative control group), rabbits treated with DDWM/BAC 95/5 (positive control group) during 12 consecutive days and rabbits treated with SD 25/75 during 28 days (Callens, 2002).

	1 h before	1 h after	n
LDH (U/l)			
Negative control	-	66 ± 20	3
DDWM/BAC 95/5	-	4823 ± 1907	3
SD 25/75	457 ± 153	810 ± 238	4
ALP (U/l)			
Negative control	-	96 ± 31	3
DDWM/BAC 95/5	-	518 ± 194	3
SD 25/75	215 ± 89	353 ± 151	4
PROTEINS (µg/ml)			
Negative control	-	627 ± 83	3
DDWM/BAC 95/5	-	7086 ± 2978	3
SD 25/75	1207 ± 262	1643 ± 310	4

Data are presented as mean ± SD.

4.2.3.1.2 Multiple administration during 5 days

During 5 consecutive days 10.0 mg of the powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 was sprayed into each nostril of the rabbit. Each day the nasal cavity was washed 1 h before and 1 h after administration. The protein concentration and enzyme activity in the nasal washing sampled 1 h before the first powder administration were considered as basal levels. Three days after the last administration, an additional washing was performed to evaluate if the release of protein, LDH and ALP had returned to their basal levels.

The LDH activity obtained 1 h and 23 h after nasal delivery of the powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 are shown in Figure 3. The LDH activity of both formulations after administration was in general significantly higher ($P < 0.05$) compared to the basal LDH activity. Figure 3 also clearly illustrates that the LDH release was higher compared to the negative control, but lower than the positive control (Callens, 2002).

Although the increase for the formulation SD 25/75 was higher compared to (SD 25/75)/$CaCO_3$ 90/10, this difference was only significant ($P < 0.05$) after the second administration because on days 3 to 5 a progressive decrease in LDH activity could be observed after nasal administration of SD 25/75. This could be due to the nasal mucosa adapting to the powders after multiple exposure. In case of the positive control no decrease in LDH activity was observed after multiple administrations.

For both powder formulations a pronounced decrease in LDH release 23 h after administration was observed which was slightly higher than the basal LDH release and which decreased with increasing administrations. Moreover, the LDH activity measured on day 8 (i.e. 3 days after the last administration) was similar ($P > 0.05$) to the basal LDH activity, indicating that any damage to the nasal epithelium induced by the powder formulations was reversible.

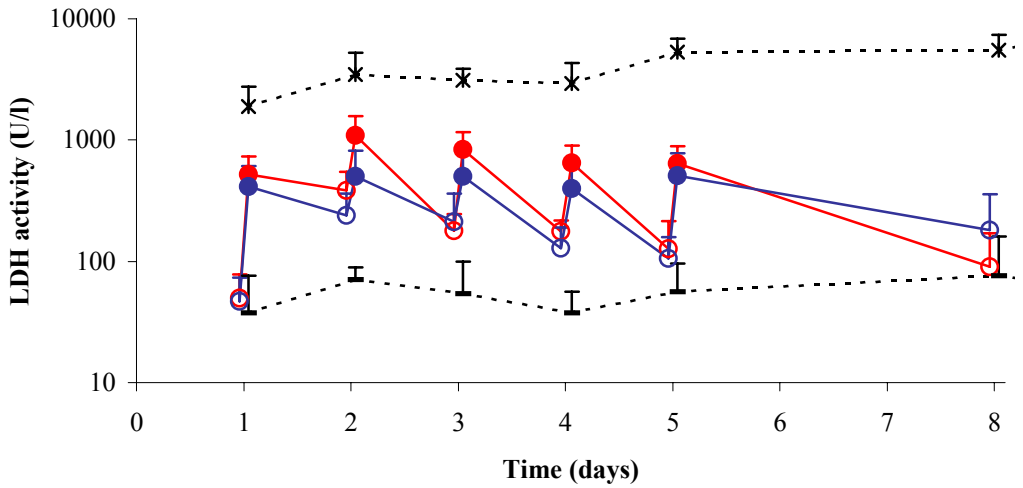

Figure 3. LDH activity in the nasal washings of rabbits 1 h before (open symbols) and 1 h after (closed symbols) administration of the powder formulations SD 25/75 (red) and (SD 25/75)/$CaCO_3$ 90/10 (blue) during 5 consecutive days (n = 6, mean ± SD). The LDH activity in the nasal washings of the negative control (untreated) (-) and positive control (DDWM/BAC 95/5) (∗) rabbits performed 1 h after administration were added for comparison (n = 3, mean ± SD) (Callens, 2002).

In Figure 4 the ALP activity in the nasal washings after nasal administration of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 are shown. 1 h after nasal administration of SD 25/75 and

(SD 25/75)/$CaCO_3$ 90/10 an increased ALP activity was measured from day 2 and day 4, respectively. Only after the second administration the ALP activity induced by SD 25/75 was higher than for (SD 25/75)/$CaCO_3$ 90/10, although not significantly ($P > 0.05$). A decreased ALP release from the mucosa was observed after repeated nasal administrations of SD 25/75, whereas for the positive control an increased was seen.

23 h after second and third administration of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10, respectively, the ALP release had decreased to levels slightly above the basal ones. For both formulations the ALP activity measured after 23 h decreased after repeated administrations and 3 days after the last administration the basal level of ALP release was detected, indicating a complete recovery of the nasal mucosa.

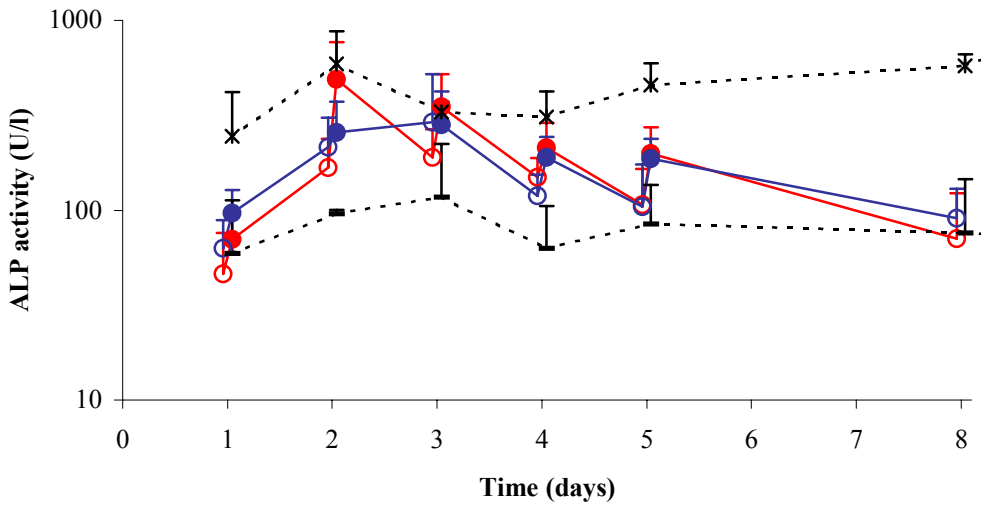

Figure 4. ALP activity in the nasal washings of rabbits 1 h before (open symbols) and 1 h after (closed symbols) administration of the powder formulations SD 25/75 (red) and (SD 25/75)/$CaCO_3$ 90/10 (blue) during 5 consecutive days (n = 6, mean ± SD). The ALP activity in the nasal washings of the negative control (untreated) (-) and positive control (DDWM/BAC 95/5) (∗) rabbits performed 1 h after administration were added for comparison (n = 3, mean ± SD) (Callens, 2002).

The protein concentration in the nasal cavity of rabbits after nasal delivery of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 are shown in Figure 5. Although no statistical differences ($P > 0.05$) were detected between the protein concentration before and after nasal delivery, a similar trend was observed as for the release of the marker enzymes. After administration a

pronounced increase in protein release was observed combined with a return to the basal levels after 23 h. In comparison with (SD 25/75)/$CaCO_3$ 90/10 the SD 25/75 powder induced a higher release of proteins from the nasal mucosa, although this difference was not significant ($P > 0.05$).

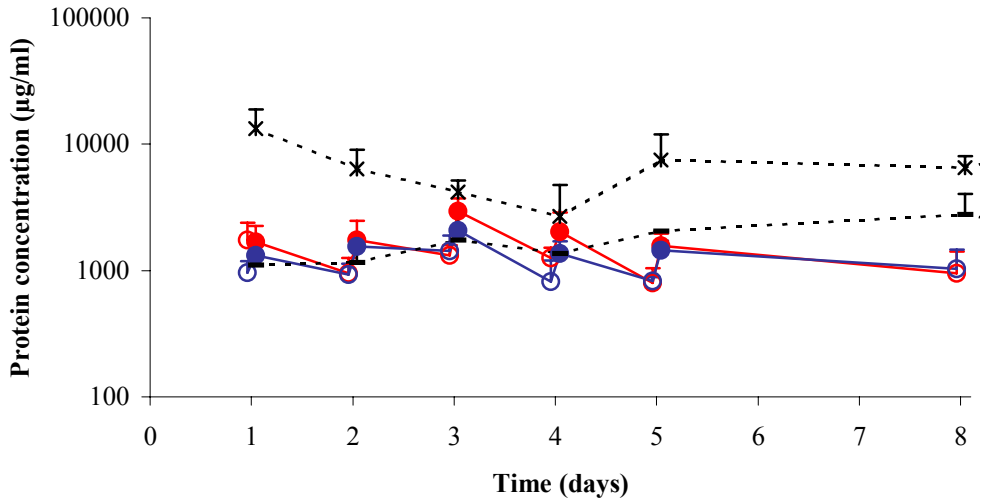

Figure 5. Protein concentration in the nasal washings of rabbits 1 h before (open symbols) and 1 h after (closed symbols) administration of the powder formulations SD 25/75 (red) and (SD 25/75)/$CaCO_3$ 90/10 (blue) during 5 consecutive days (n = 6, mean ± SD). The protein concentration in the nasal washings of the negative control (untreated) (-) and positive control (DDWM/BAC 95/5) (∗) rabbits performed 1 h after administration were added for comparison (n = 3, mean ± SD) (Callens, 2002).

4.2.3.2 Slug Mucosal Irritation assay

The mucosal tolerance of the powders SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 was also evaluated using an alternative mucosal irritation test on slugs. The irritation potency was evaluated based on the amount of mucus produced by the slugs, whereas tissue damage was assessed by the release of proteins and enzymes (LDH and ALP) from the body wall. The observations made during this study will be compared to the results from the non-invasive washing method in rabbits (cfr. paragraph 4.2.3.1). In addition, the results of preliminary tests performed in order to optimise the procedure of the mucosal irritation test on slugs are discussed.

The results of the mucosal irritation of powders tested according to the procedure described by Callens et al. (2001) (Exp. I) are shown in Table 3.

Table 3. Effect of SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 on the endpoints of the Slug Mucosal Irritation test (n = 5, mean ± SD).

	Total MP (%)	Protein (µg/ml.g)	LDH (U/l.g)	ALP (U/l.g)	(n)	**Irritation/ Damage**
Exp I						
DDWM	4.5 ± 1.1	10 ± 7	-	-	0/5	**NI/Min**
DDWM/SLS 80/20	23.3 ± 1.4	120 ± 28	0.3 ± 0.2	0.2 ± 0.5	1/5	**Sev/Sev**
SD 25/75	18.4 ± 4.2	49 ± 30	0.2 ± 0.3	-	0/5	**Mo/Mild**
(SD 25/75)/$CaCO_3$ 90/10	16.1 ± 2.0	30 ± 13	-	-	0/5	**Mo/Mild**
Exp II						
DDWM	5.5 ± 1.8	36 ± 16	0.3 ± 0.2	-	0/5	**NI/Mild**
DDWM/SLS 80/20	21.1 ± 1.0	150 ± 86	2.1 ± 1.8	0.4 ± 0.6	2/5	**Sev/Sev**
SD 25/75	18.6 ± 1.1	61 ± 40	0.5 ± 0.4	-	0/5	**Mo/Mo**
(SD 25/75)/$CaCO_3$ 90/10	16.8 ± 1.8	26 ± 15	0.2 ± 0.2	-	0/5	**Mo/Mild**
Exp III						
DDWM	6.8 ± 1.4	19 ± 7	0.1 ± 0.1	-	0/5	**NI/Min**
DDWM/SLS 80/20	19.6 ± 2.4	175 ± 46	2.5 ± 0.6	0.4 ± 0.4	5/5	**Mo/Sev**
SD 25/75	17.9 ± 1.4	88 ± 32	1.2 ± 1.2	0.3 ± 0.6	1/5	**Mo/Mo**
(SD 25/75)/$CaCO_3$ 90/10	16.2 ± 1.3	48 ± 21	0.4 ± 0.4	-	0/5	**Mo/Mild**

MP: mucus production in % (w/w) of the body weight

The powder formulations induced a mucus production higher than the negative control but lower than the positive control. After contact with SD 25/75 the slugs produced a slightly higher amount of mucus compared to (SD 25/75)/$CaCO_3$ 90/10. The amount of mucus produced per day by the positive control slugs decreased after repeated treatments, whereas the mucus production of the slugs treated with the test formulations remained constant. This indicated that the mucus-secreting cells of the positive control slugs were severely and irreversibly damaged after repeated contact. In contrast, the cells of slugs treated with SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 sufficiently recovered in between contact periods.

The protein release after contact with SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 was also higher than for the negative control. For SD 25/75 the release was slightly higher than for (SD 25/75)/$CaCO_3$ 90/10 and seemed to increase after repeated contact with the material. This indicated that SD 25/75 might have a higher membrane damaging potential. However, the protein concentrations in the PBS samples of the positive control slugs were clearly higher and the release increased after each contact period.

The LDH activity of the negative control slugs and the slugs treated with (SD 25/75)/$CaCO_3$ 90/10 was below the detection limit. In case of SD 25/75 LDH was only detected on the last day of the experiment for 3 slugs. After contact with the positive control all slugs released LDH, three of them already after the third treatment. ALP release was only detected in samples of the positive control slugs.

Based on the endpoints of the SMI assay summarised in Table 3 and the prediction model which is shown in Figure 2, the powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 are classified according to their irritation potency and membrane damaging capacity. Despite the higher mucus production, protein concentration and LDH activity after contact with SD 25/75, both formulations were considered to be moderately irritating and mildly membrane damaging. The negative control was classified as a non-irritant inducing minimal damage to membranes. In contrast, the positive control was a severe irritant causing severe damage of mucosal tissue after contact.

The following experiments were performed in order to evaluate if incomplete removal of the polymer film formed during the 30-min contact period between mucus and the test formulation may act as a barrier to protein and enzyme release from the mucosal tissue of the slug into the PBS solution. Therefore, the procedure of the SMI assay was modified. A first experiment was performed wherein the foot mucosa of the slug was washed with 2 ml PBS and cautiously dried with paper immediately after the 30-min contact period (Exp. II). In a second experiment the foot mucosa was carefully cleaned with paper moistened with PBS (Exp. III). The results of both experiments are shown in Table 3.

Using the procedure of Exp. II and III a similar total mucus production was measured compared to Exp. I, resulting in the same classification for irritation according to the prediction model. Using the procedure of Exp. III the positive control was classified as

moderate irritating instead of severe irritating as was concluded in Exp. I and II. However, it should be mentioned that the positive control in Exp. III (19.6 % mucus production) was a borderline case as the upper limit of moderate irritation for mucus production was 20 %.

The protein concentration in the PBS samples using the protocol of Exp. II was higher compared to the concentrations measured in Exp. I, even for the negative control. Protein concentrations in the PBS samples obtained using Exp. III were higher compared to Exp. II (except for the negative control).

Using the modified test set-ups (Exp. II and III) LDH release was detected for all formulations. For the negative control samples LDH was measured earlier and in higher activities in Exp. II compared to Exp. III. This observation was in contradiction with the results of the positive control and the two test formulations. For the positive control and SD 25/75 higher LDH activities were detected at an earlier stage when the modified protocols were used. During Exp. III LDH was detected in all slugs treated with the positive control released from the third contact period, whereas in Exp. II there was still one slug for which LDH activity was found only after the last contact period. In case of SD 25/75 LDH activities were found after the fourth contact period in two and four slugs during Exp. II and III, respectively. Also for (SD 25/75)/$CaCO_3$ 90/10 LDH release occurred earlier and in higher activities in Exp. III: three slugs produced LDH after four days in Exp. III, whereas in Exp II only two slugs released LDH after the fifth contact period.

The ALP release of the positive control slugs showed a similar trend between the different procedures as the LDH release: an earlier detection, in higher quantities and by a higher number of slugs in the following rank order Exp. III > Exp. II > Exp. I. The ALP release seen for SD 25/75 in Exp III. was produced by one slug from the third contact period.

Due to the higher protein concentrations in the PBS samples of Exp. II, the membrane damaging potential of the negative control formulation was classified in a higher scale compared to Exp. I: mild tissue damaging (Exp. II) versus minimal (Exp. I). Using the procedure of Exp. III the same prediction was made for the negative control as in Exp. I: minimal membrane damage.
Changing the protocol of the SMI test had no influence on the prediction of the positive control: severe tissue damage in all cases.

Despite the higher protein concentration and LDH activity measured in Exp. II and III for (SD 25/75)/$CaCO_3$ 90/10, this material was classified in the same class as in Exp. I: mild tissue damaging. On the other hand the SD 25/75 formulation, was more membrane damaging following the modified procedures compared to Exp I: moderate (Exp. II and III) versus mild tissue damage (Exp. I). However, it should be noted that SD 25/75 in Exp. I was a borderline case for its membrane damaging potential: a mean protein release of 49 ± 30 µg/ml.g was measured, whereas the lower limit of the moderate irritation class was 50 µg/ml.g.

4.2.4 Discussion

It has been demonstrated that the non-invasive washing technique in rabbits (Callens et al., 2001) is a reproducible method to evaluate the toxicity of nasal formulations. The simplicity of the experimental set-up is in contrast with other in vivo methods described in literature. Hirai et al. (1981) and Huang et al. (1985) developed an in-situ perfusion technique whereby the trachea of anaesthetised rats was cannulated to maintain respiration. A second cannula was inserted through the oesophagus to the posterior part of the nasal cavity. This cannula served to perfuse the nasal cavity with the test solution, which was recirculated during 1.5 to 6 h using a peristaltic pump. Furthermore, the passage of the nasopalatine tract was sealed with an adhesive agent to prevent drainage of the solution from the nasal cavity to the mouth. This method was adapted by Marttin et al. (1995) in order to better mimic the physiological conditions. However, cannulation of anaesthetised rats was still required. Moreover, in perfusion tests the mucus layer is progressively washed away, hence the nasal epithelium is in direct contact with the test solutions (Merkus et al., 1999).

Using the non-invasive washing technique in rabbits it was shown that both powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 caused mucosal irritation and membrane damage after nasal administration as an increased protein, LDH and ALP release from the nasal mucosa was observed. A higher membrane damaging potential was observed for SD 25/75 compared to (SD 25/75)/$CaCO_3$ 90/10. For both powders the effects on the epithelium were reversible as the protein concentrations and enzyme activities decreased within 24 h, returning to their basal levels within 3 days.

A progressive decrease in protein and enzyme release was observed after repeated administrations to rabbits. This might be related to adaptation of the nasal mucosa to the powders after multiple exposure. This might also be attributed to incomplete clearance of the formulation from the nasal cavity within 24 h (Callens et al., 2003). As a result a polymer film covering the nasal mucosa would prevent contact between the nasal epithelium and powder particles after the next powder administration. In addition a polymer film would also prevent the release of protein or marker enzymes into the nasal cavity. Therefore, the toxicological effects induced by the formulation may be underestimated. However, the non-invasive washing technique in rabbits allowed a good prediction of the membrane damaging potential of the powders as the conclusions correlated with those drawn with the SMI assay.

Using the Slug Mucosal Irritation test the formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 induced a higher mucus production and release of proteins compared to the negative control formulation, indicating that the formulations had a membrane irritating and damaging potential, although considerably lower compared to the positive control. Only for SD 25/75 LDH release was detected in Exp. I. In the modified procedures, LDH release was observed for both formulations, but the activities measured for SD 25/75 were the highest. Using the prediction model SD 25/75 was also classified as having a higher membrane damaging potential compared to (SD 25/75)/$CaCO_3$ 90/10.

The protocols of the SMI assay had a pronounced influence on the protein concentration and LDH and ALP activity found in the PBS samples: the results obtained using the modified protocols (Exp. II and III) suggested that the procedure of Exp I may underestimate the tissue damaging potency of the formulations, especially for test substances on the borderline between the mild and moderate class. This might be explained by a partial removal of the thin polymer film covering the foot mucosa of the slug, hampering the release of proteins and enzymes from the epithelial cells. In Exp II the negative control (DDWM) was classified as mild instead of minimal damaging for the membranes. However, the non-irritating properties of DDWM have clearly been demonstrated by several authors (Bottenberg et al., 1991; Bouckaert et al., 1993, 1996; Callens et al., 2001; Ceulemans et al., 2001; Weyenberg et al., 2004), suggesting that the results obtained with Exp. II overestimate the mucosal toxicity induced by dry powder formulations.

Although the same conclusions were drawn for the test formulations with Exp. II and Exp. III, the procedure of Exp. II is considered to be more aggressive as the protein release

for the negative control slugs was higher than the results in Exp. I and LDH release was detected in higher number of slugs in comparison with Exp. III.
Consequently, the procedure of Exp III will be further optimised and validated to allow a more accurate prediction of the mucosal toxicity of dry powder formulations using slugs as test organism.

The moderate irritating potential and mild to moderate membrane damage of the powders SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 is probably due to poly(acrylic acid) incorporated in the formulation as Carbopol® has been reported to cause membrane damage when used in high concentrations. Buccal bioadhesive tablets containing ≥ 50 % Carbopol® 934P induced mucosal injuries in humans (Bottenberg et al., 1991). Nasal delivery of Carbopol® 971P to rabbits resulted in severe inflammation of the nasal epithelium (Ugwoke et al., 2000). However, in the present study the spray-dried mixture of Amioca®starch and Carbopol® 974P was neutralised, thus reducing the irritating and membrane damaging potential of Carbopol® 974P. Previous experiments have shown (Adriaens et al., 2003) that slugs exposed to a non-neutralised mixture of starch/Carbopol® 974P (ratio: 60/40, w/w) produced a similar amount of mucus after multiple contact periods compared to slugs treated with a neutralised mixture of starch/Carbopol® 974P containing 75 % Carbopol®. In addition, the non-neutralised mixture containing 40 % Carbopol® 974P induced a cytosolic LDH release (0.70 ± 0.85 U/l.g). Under the same experimental conditions only a minimal LDH release (0.2 ± 0.3 U/l.g) was seen for the neutralised mixture containing 75 % Carbopol®. According to the prediction model a neutralised mixture of starch/Carbopol® 974P at a ratio of 25/75 has a similar mucosal toxicity as a non-neutralised starch/Carbopol® 974P mixture containing 40 % Carbopol®: moderate irritation and mild membrane damage.

4.2.5 Conclusions

A good correlation was found between the non-invasive nasal washing technique in rabbits and the Slug Mucosal Irritation assay during the toxicological evaluation of the nasal powder formulations SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10. As a slightly higher mucosal tolerance was obtained for the formulation containing $CaCO_3$, the absorption enhancement of insulin

observed with the carrier (SD 25/75)/$CaCO_3$ 90/10 was not due to a higher disruption of the integrity of the mucosal tissue.

Both formulations were classified as substances which cause moderate membrane irritation and mild to moderate membrane damage, hence they are not recommended for chronic drug therapies, but are rather indicated for single use drug delivery (e.g. nasal vaccination).

4.2.6 References

Adriaens E., Remon J.P., Gastropods as an evaluation tool for screening the irritating potency of absorption enhancers and drugs. Pharm. Res. 16 (1999) 1239-1243.

Adriaens E. and Remon J.P., The evaluation of an alternative mucosal irritation test using slugs. Tox. Appl. Pharm. 182 (2002) 169-175.

Adriaens E., Ameye D., Dhondt M.M.M., Foreman P., Remon J.P., Evaluation of the mucosal irritation potency of co-spray dried Amioca®/poly(acrylic) and Amioca®/Carbopol® 974P mixtures. J. Contr. Rel. 88 (2003) 393-399.

Agu R.U., Jorissen M., Willems T., Van den Mooter G., Kinget R., Augustijns P., Effects of pharmaceutical compounds on ciliary beating in human nasal epithelial cells: a comparative study of cell culture models. Pharm. Res. 16 (1999) 1380-1385.

Agu R.U., Jorissen M., Kinget R., Verbeke N., Augustijns P., Alternatives to in vivo nasal toxicological screening for nasally administered drugs. STP Pharm. Sci. 12 (2002) 13-22.

Bottenberg P., Cleymaet R., Demuynck C., Remon J.P., Coomans D., Michotte Y., Slop D., Development and testing of bioadhesive, fluoride-containing slow-release tablets for oral use. J. Pharm. Pharmacol. 43 (1991) 457-464.

Bouckaert S., Lefebre R.A., Colardyn F., Remon J.P., Influence of application site on bioadhesion and slow-release characteristics of a bioadhesive buccal slow-release tablet of miconazole. Eur. J. Clin. Pharmacol. 44 (1993) 331-335.

Bouckaert S., Vakaet L., Remon J.P., Influence of the buccal application site of a bioadhesive slow-release tablet on salivary of miconazole concentrations in irradiated patients. Int. J. Pharm. 130 (1996) 257-260.

Callens C., Adriaens E., Dierckens K., Remon J.P., Toxicological evaluation of a bioadhesive nasal powder containing a starch and Carbopol® 974P on rabbit nasal mucosa and slug mucosa. J. Control. Rel. 76 (2001) 81-91.

Callens C., Ontwikkeling van een platform voor nasale toediening van peptiden. Doctoral thesis, Ghent University, Belgium, 2002.

Callens C., Pringels E., Remon J.P., Influence of multiple nasal administrations of bioadhesive powders on the insulin bioavailability. Int. J. Pharm. 250 (2003) 415-422.

Ceulemans J., Vermeire A., Adriaens E., Remon J.P., Ludwig A., Evaluation of a mucoadhesive tablet for ocular use. J. Control. Release. 77 (2001) 333-344.

Dhondt M.M.M., Adriaens E., Remon J.P., The Evaluation of the local tolerance of vaginal formulations, with or without Nonoxynol-9, using the Slug Mucosal Irritation test. Sex. Trans. Dis. 31 (2004) 229-235.

Dhondt M.M.M., Adriaens E., Van Roey J., Remon, J.P., Evaluation of the local tolerance of vaginal formulations containing TMC 120 using rabbits and slugs. Eur. J. Pharm. Biopharm. 60 (2005) 419-425.

Harvey M.D., Bablekis V., Banks P.R., Skinner C.D., Utilization of the non-covalent fluorescent dye, NanoOrange, as a potential clinical diagnostic tool - Nanomolar human serum albumin quantitation. J. Chromatogr. B 754 (2001) 345-356.

Hirai S., Yashiki T., Mima H., Absorption of drugs from the nasal mucosa of rat. Int. J. Pharm. 7 (1981) 317-325.

Huang C.H., Kimura R., Bawarashi R., Hussain A., Mechanism of nasal absorption of drugs. I. Physicochemical parameters influencing the rate of in situ nasal absorption of drugs in rats. J. Pharm. Sci. 74 (1985) 608-611.

Krishnamoorthy R., Wolka A.M., Shao Z., Mitra A.K., Cyclodextrins as mucosal absorption promoters. IV. Evaluation of nasal mucotoxicity. Eur. J. Pharm. Biopharm. 41 (1995) 296-301.

Longenecker J.P., Moses A.C., Flier J.S., Silver R.D., Carey M.C., Dubovi E.J., Effects of sodium taurodihydrofusidate on nasal absorption of insulin in sheep. J. Pharm. Sci 76 (1987) 351-355.

Marttin E., Verhoef J.C., Romeijn S.G., Merkus F.W.H.M., Effects of absorption enhancers on rat nasal epithelium in vivo: release of marker compounds in the nasal cavity. Pharm. Res., 12 (1995) 1151-1157.

Merkus F.W.H.M., Schipper N.G.M., Hermens W.A.J.J., Romeijn S.G., Verhoef J.C., Absorption enhancers in nasal drug delivery: efficacy and safety. J. Contr. Rel. 24 (1993) 201-208.

Merkus F.W.H.M., Verhoef J.C., Marttin E., Romeijn S.G., van der Kuy P.H.M., Hermens W.A.J.J., Schipper N.G.M., Cyclodextrins in nasal drug delivery. Adv. Drug Deliv. Rev. 36 (1999) 41-57.

Mishima M., Wakita Y., Nakano M., Studies on the promoting effects of medium chain fatty acid salts on the nasal absorption of insulin in rats. J. Pharmacobio-Dyn. 10 (1987) 624-631.

Schipper N.G.M., Verhoef J.C., Merkus F.W.H.M., The nasal mucociliary clearance: relevance to nasal drug delivery. Pharm. Res. 8 (1991) 807-814.

South A., Structure of the integument. In: South A. (Ed.), Terrestrial slugs: biology, ecology and control. Chapman and Hall, London (1992) 37-43.

Sørensen A. Animal models for buccal and nasal delivery studies. In: Duchêne D. (Ed.), Buccal and nasal administration as an alternative to parenteral administration. European Symposium, Paris, 10-11 Dec, Editions de Santé (1991) 162-173.

Ugwoke M.I., Agu R.U., Jorissen M., Augustijns P., Sciot R., Verbeke N., Kinget R., Nasal toxicological investigations of Carbopol® 971P formulation of apomorphine: Effects on ciliary beat frequency of human nasal primary cell culture and in vivo on rabbit nasal mucosa. Eur. J. Pharm. Sci. 9 (2000) 387-396.

Weyenberg W., Vermeire A., Dhondt M.M.M, Adriaens E., Kestelyn P., Remon J.P., Ludwig, A., Ocular bioerodible minitablets as strategy for the management of microbial keratitis. Investigative Ophthalmology & Visual Science. 45 (2004) 3229-3233.

Chapter 5 Stability of spray-dried Amioca® starch/Carbopol® powder

5.1 Introduction

Stability testing was performed on a spray-dried mixture of Amioca® starch/Carbopol® 974P (ratio: 25/75, w/w) stored during 9 months as bulk powder in an open container at different temperatures and relative humidities. The moisture content, simulated nasal fluid uptake rate and rheological properties of the powder as well as the pharmacokinetic parameters of insulin after nasal delivery to rabbits were determined in function of time.

5.2 Materials and Methods

5.2.1 Materials

Actrapid® HM 100 (100 IU/ml) (human monocomponent insulin) was obtained from Novo-Nordisk (Bagsvaerd, Denmark). The spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75, w/w) (SD 25/75) (batch number: 12621: 14F) was prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Carbopol® 974P was supplied by Noveon (Cleveland, Ohio, USA). All other chemicals were of analytical grade.

5.2.2 Storage conditions

SD 25/75 was stored as bulk powder in an open container at three different storage conditions: at room temperature (25 ± 2 °C) in a sealed box containing silica particles to obtain a relative humidity of 10 ± 5 %, at room temperature (25 ± 2 °C) in a sealed box containing a saturated sodium bromide solution to obtain a relative humidity of 60 ± 5 % and

in an oven (40 ± 2 °C) in a sealed box containing a saturated sodium chloride solution to obtain a relative humidity of 75 ± 5 %.

5.2.3 Insulin formulations

5.2.3.1 Insulin solution for intravenous administration

An insulin solution of 0.8 IU/ml was prepared by diluting Actrapid® HM 100 in a phosphate buffered saline solution (PBS, pH 7.4) (2.38 g $Na_2HPO_4.2H_2O$, 0.19 g KH_2PO_4 and 8.0 g NaCl per liter distilled water) of which 0.5 ml was administered intravenously to rabbits (n = 10).

5.2.3.2 Nasal powder formulations

After dispersion of 0.5 g SD 25/75 in 15 ml distilled water, 2.0 M NaOH was added until pH 7.4 was reached. Then the insulin solution (Actrapid® HM 100) was added in order to obtain a final concentration of 1 IU insulin per mg powder, taking the moisture content of the powder formulation into account.
To obtain a powder, the aqueous dispersion was freeze-dried using an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to 228 K within 175 min at 1000 mbar. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (283 K) and reduced pressure (0.1 - 0.2 mbar) for 7 h.
After freeze-drying the powder was sieved (63 µm) at low relative humidity (20 %) and ambient temperature. The fraction below 63 µm was stored in a desiccator at 4 - 8 °C until use.

5.2.4 Measurements

The nasal bioavailability, the simulated nasal fluid uptake rate, the moisture content and the rheological properties were determined before storage and after 3, 6 and 9 months storage

(25 °C - 10 % RH; 25 °C - 60 % RH and 40 °C - 75 % RH). A freeze-dried formulation (cfr. paragraph 5.2.3.2) was used to determine the nasal bioavailability, liquid uptake rate and rheological properties, whereas the moisture content of the bulk powders was determined.

5.2.5 Moisture content

The moisture content was determined using a Mettler DL 35 Karl Fischer titrator (Mettler-Toledo, Beersel, Belgium). Hydranal®-Composite 2 (Riedel-de Haën Laborchemicalien, Seelze, Germany) was used as titrant and anhydrous methanol (Riedel-de Haën Laborchemicalien, Seelze, Germany) as reaction medium. Calibration and verification of the titrant (theoretical 2 mg H_2O/ml) was performed with distilled water.

5.2.6 Liquid uptake rate

The liquid uptake rate was studied hydrodynamically. 50 mg powder formulation was placed on the upper side of a filter connected to a reservoir filled with simulated nasal fluid (SNF) (7.45 mg/ml NaCl, 1.29 mg/ml KCl and 0.32 mg/ml $CaCl_2$, Melon, 1968). The measurements were performed at 32 ± 0.5 °C. The amount of SNF absorbed was determined volumetrically in function of time.

5.2.7 Rheological properties

The elasticity (G') and viscosity (G") of the powder formulations were determined on a TA Instruments AR 1000-N Rheometer (Zellik, Belgium) after dispersion (10 %, w/w) in simulated nasal fluid. The measurements were performed at 32 ± 0.5 °C using a cone of 4 cm having an angle of 1° and applying an oscillation stress of 25 Pa and a frequency of 0.1 Hz.

5.2.8 Nasal bioavailability

New Zealand white rabbits (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available ad libitum. They were sedated with an intramuscular injection of 0.05 ml/kg Placivet® (Codifar, Florida, USA). The rabbits received 0.4 IU insulin intravenously. 10 mg powder formulation (equivalent to 10 IU insulin) was administered in each nostril using polyethylene tubes (Medisize, Hillegom, The Netherlands). The powder was released from the tubes using a syringe containing 1 ml compressed air (2.5 bar). This device was based on a system developed by Sørensen (1991). The tubes were filled under conditions of low relative humidity (20 %) and ambient temperature. Blood samples were collected from the ear veins at -5, 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after intravenous administration and at -5, 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min after nasal delivery of the powder formulations. The samples were centrifuged (700g, 5 min) and the sera were frozen at –20 °C until RIA-analysis (Coat-A-Count® kit, DPC, Humbeek, Belgium). The radioactivity of the samples was quantified using a Cobra gamma counter (Canberra Packard Benelux, Zellik, Belgium). The individual serum concentration-time profiles were analysed using MW/Pharm version 3.15 (Medi-ware, Utrecht, The Netherlands). For calculation of the absolute biovailability (BA) the weight of the rabbits and the concentration of insulin in the powder formulations were taken into account. The maximum insulin serum concentrations (C_{max}) and t_{max} values were determined from the individual serum concentration-time profiles.

- *Statistical analysis*

Statistical analysis was performed on the absolute bioavailability, C_{max} and t_{max} values of the different powder formulations using a one-way Anova followed by post hoc Scheffé test with $P < 0.05$ as significance level. The data and residuals were tested for normal distribution using the Kolmogorov-Smirnov test and the homogeneity of variances was tested using the Levene's test. If the distribution of the data or residuals was not normal or the variances were not homogeneous, the data were transformed to their power. The computer software SPSS version 11.0 was used for statistical analysis.

5.3 Results and Discussion

5.3.1 Moisture content

In Figure 1 the moisture content of the SD 25/75 powder in function of storage time and storage conditions is shown.

SD 25/75 stored at 10 % RH resulted in a lower moisture content compared to the initial spray-dried powder due to the water absorbing capacity of the silica particles. Storage at 60 % and 75 % RH increased the water content from 4 % (t = 0) to 10 and 12 % (w/w), respectively.

Storage time had no influence on the moisture content at each storage condition.

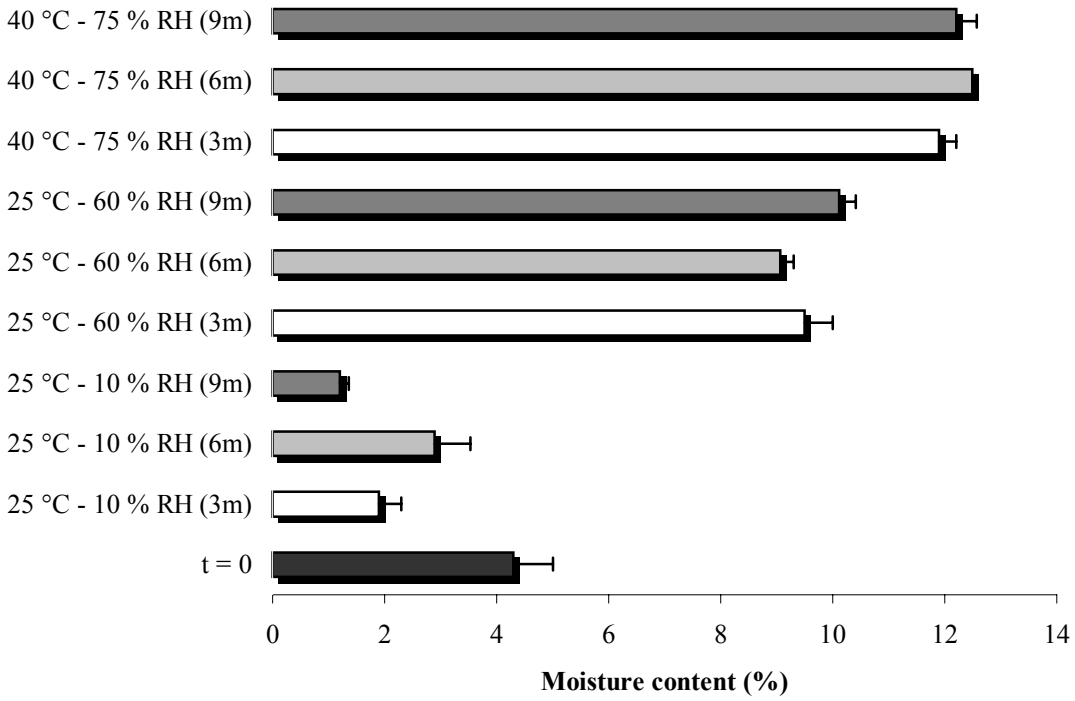

Figure 1. Influence of storage time and storage conditions on moisture content (% (w/w)) of SD 25/75 (mean ± SD, n = 3).

5.3.2 Liquid uptake

The uptake of simulated nasal fluid (SNF) by the freeze-dried nasal powders based on SD 25/75 stored at different conditions is given in Figure 2.

A higher relative humidity and temperature induced a fast hydration rate: at 40 °C – 75 % RH a fast and complete hydration of the polymer chains (SNF uptake: ± 7.7 ml/g) was observed within 45 s. Storage at 25 °C – 10 % RH and 25 °C – 60 % RH resulted in nasal powder formulations having a slower SNF uptake: ± 12.6 ml/g and ± 16.0 ml/g over a period of 1 h, respectively. The hydration rate of powder stored at 25 °C – 10 % RH was similar to the hydration rate prior to storage and was slightly lower than the powder stored at 25 °C – 60 % RH. Storage time had no influence on the hydration rate of the formulation.

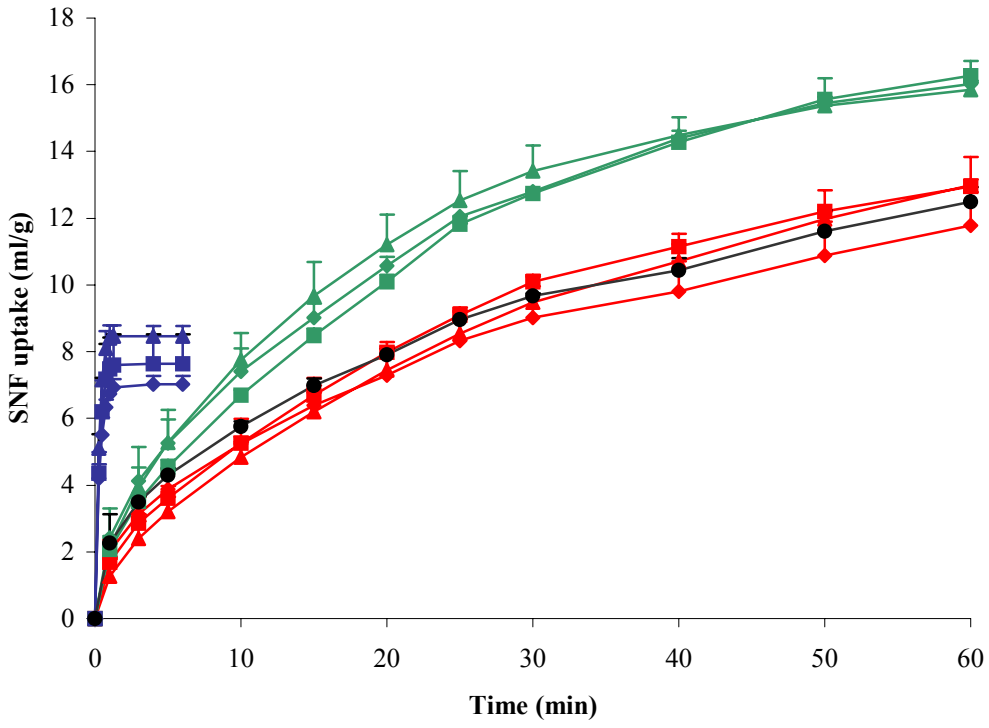

Figure 2. Influence of storage time (0m (●), 3m (▲), 6m (■) and 9m (◆)) and storage conditions (25 °C – 10 % RH (red), 25 °C – 60 % RH (green) and 40 °C – 75 % RH (blue)) on uptake of simulated nasal fluid by SD 25/75 (mean ± SD, n = 2).

5.3.3 Rheological properties

The viscosity and elasticity after dispersion in simulated nasal fluid of SD 25/75 powders stored at different conditions are shown in Table 1.

The elasticity (G') and viscosity modulus (G") of the SD 25/75 powders stored at 25 °C - 10 % RH did not alter during storage and was slightly lower compared to the data obtained after storage at 25 °C - 60 % RH. The differences in G' and G" between the sample time points were minimal.

On the other hand, storage at elevated temperature (40 °C) and relative humidity (75 %) resulted in a high viscosity and elasticity after dispersion into SNF. After 6 months the elasticity modulus was strongly decreased, while the viscosity modulus remained similar. After 9 months it was not possible to define G' and G" due to problems of homogeneity of the gel.

Table 1. Influence of storage time and storage conditions of SD 25/75 on elasticity (G') and viscosity (G") after dispersion into simulated nasal fluid (mean ± SD, n = 3).

Formulation	G' (Pa)	G" (Pa)
reference (t = 0)	2962 ± 64	249 ± 26
25 °C – 10 % RH (3 m)	2815 ± 68	258 ± 27
25 °C – 10 % RH (6 m)	2378 ± 75	260 ± 4
25 °C – 10 % RH (9 m)	2078 ± 84	235 ± 13
25 °C – 60 % RH (3 m)	3346 ± 203	295 ± 13
25 °C – 60 % RH (6 m)	3078 ± 214	284 ± 14
25 °C – 60 % RH (9 m)	3387 ± 82	293 ± 7
40 °C – 75 % RH (3 m)	20315 ± 1151	3695 ± 273
40 °C – 75 % RH (6 m)	7897 ± 455	3577 ± 128
40 °C – 75 % RH (9 m)	-	-

5.3.4 Nasal bioavailability

The influence of storage time and storage conditions on the absolute bioavailability, C_{max} and t_{max} of insulin obtained after nasal delivery to rabbits is shown in Table 2. The insulin serum concentration-time profiles are depicted in Figure 3.

It can be observed that the absorption of insulin was not influenced after storage at 25 °C – 10 % RH as similar serum insulin concentration-time profiles were obtained during the entire test period ($P > 0.05$). As described earlier (cfr. paragraph 5.3.2 and 5.3.3) the physical characteristics of the powder did not change during storage under these conditions.

Table 2. Influence of storage time and storage conditions of SD 25/75 on the pharmacokinetic parameters of insulin after nasal delivery to rabbits (mean ± SD).

Formulation	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
reference (point t = 0)	12.4 ± 2.0	441 ± 125	36.9 ± 5.6	8
25 °C – 10 % RH (3 m)	11.4 ± 5.0	389 ± 176	44.4 ± 10.9	8
25 °C – 10 % RH (6 m)	10.2 ± 5.2	334 ± 192	38.9 ± 7.2	8
25 °C – 10 % RH (9 m)	11.6 ± 4.3	398 ± 191	40.1 ± 6.5	7
25 °C – 60 % RH (3 m)	17.0 ± 5.6	677 ± 244	39.1 ± 5.1	8
25 °C – 60 % RH (6 m)	16.4 ± 4.4	781 ± 321	35.6 ± 7.3	8
25 °C – 60 % RH (9 m)	17.9 ± 7.0	741 ± 420	41.6 ± 6.3	6
40 °C – 75 % RH (3 m)	20.9 ± 7.7	1354 ± 383 ***	23.2 ± 8.2 **	7
40 °C – 75 % RH (6 m)	3.7 ± 1.1 ***	201 ± 97 *	14.6 ± 3.1 ***	8
40 °C – 75 % RH (9 m)	5.2 ± 2.6 *	301 ± 164	13.6 ± 2.5 ***	8

Significantly different from reference formulation (* = $0.05 \geq P > 0.01$; ** = $0.01 \geq P > 0.001$ and *** = $P \leq 0.001$)

Increasing the relative humidity from 10 to 60 % resulted in a higher absorption of insulin: an absolute bioavailability of 12.4 ± 2.0 % and 17.0 ± 5.6 % before storage and after 3 months,

respectively. This can be explained by the higher viscosity and elasticity of the formulation after dispersion into the nasal cavity and its slightly higher water absorbing capacity (cfr. paragraph 5.3.2 and 5.3.3). After 6 and 9 months the absorption of insulin was similar to the values obtained after 3 months.

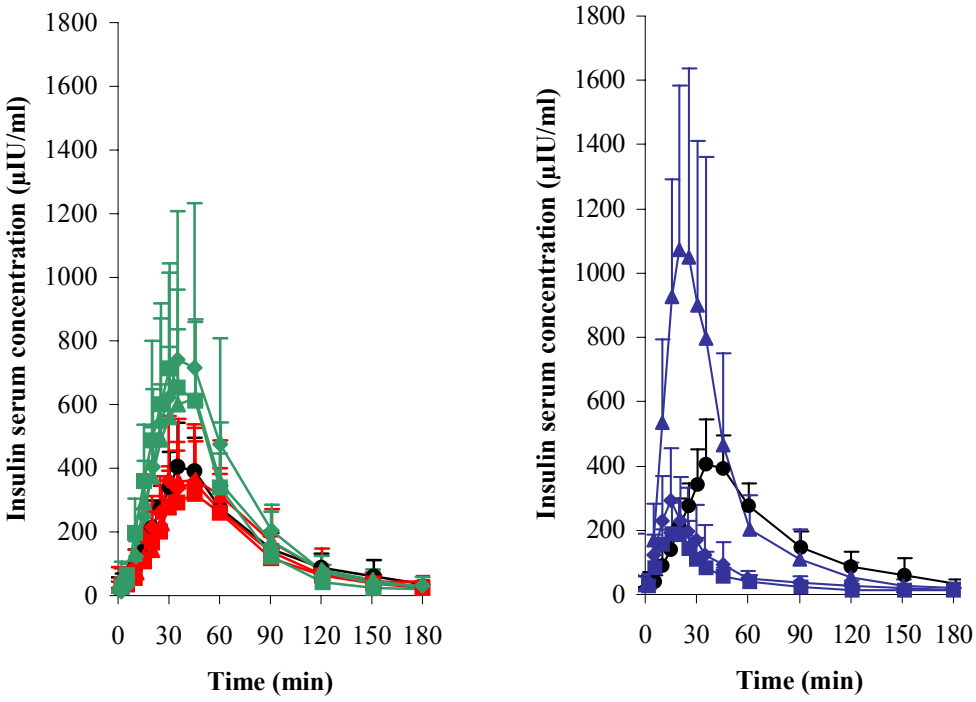

Figure 3. Influence of storage time (before (●), 3m (▲), 6m (■) and 9m (◆)) and storage conditions of SD 25/75 (25 °C – 10 % RH (red), 25 °C – 60 % RH (green) and 40 °C – 75 % RH (blue)) on the serum insulin concentration-time profiles after nasal delivery to rabbits.

Nasal delivery of SD 25/75 exposed to 75 % RH at 40 °C during 3 months resulted in a higher absorption rate of insulin. C_{max} increased significantly ($P \leq 0.001$) from 441 ± 125 µIU/ml to 1354 ± 383 µIU/ml, while t_{max} significantly ($P \leq 0.01$) decreased from 36.9 ± 5.6 min to 23.2 ± 8.2 min. Storage at these conditions during 6 and 9 months resulted in a dramatic decrease of the nasal bioavailability of insulin ($P \leq 0.001$).

SD 25/75 particles stored at 40 °C - 75 % RH absorbed water during storage (cfr. paragraph 5.3.1) whereby the polymers become in a relaxed state. As freeze-drying is

required to prepare the nasal formulations, it is hypothesised that after freeze-drying the dehydrated polymers preserve their relaxed structure. This would allow fast hydration upon contact with water (cfr. paragraph 5.3.2), thereby creating a kind of super slurper. The increased absorption observed after 3 months storage at 40 °C - 75 % RH can be explained by the fast hydration of the powder upon contact with the nasal mucosa, thereby shrinking the epithelial cells and opening the tight junctions (Edman et al., 1992; Illum et al., 1994).

Although SNF absorption of the powders stored at 40 °C - 75 % RH was independent of storage time (cfr. paragraph 5.3.2) there was a significant decrease of nasal bioavailability ($P \leq 0.001$) after 6 and 9 months storage. This is an indication that conformational changes in the polymer structure occurred in function of storage time at these conditions. Those structural changes seemed to influence the elastic properties of SD 25/75 as the elasticity decreased after dispersion of the powder stored during 6 months at 40 °C - 75 % RH, while the viscosity did not change compared to a sample stored during 3 months at the same conditions (cfr. paragraph 5.3.3). Based on these data the elasticity of the formulation can also be considered an important factor in drug absorption. However, the exact mechanism of how the elasticity modulus of the carrier can modulate the absorption of insulin remains unclear. On the one hand the decreased elasticity of SD 25/75 after 6 months storage at 40 °C - 75 % RH (compared to storage during 3 months) may result in a faster clearance of the formulation from the nasal cavity. As a result the residence time in the nasal cavity would decrease, possibly contributing to the lower nasal bioavailability of insulin. On the other hand it should be noticed that the same formulation had a higher elasticity compared to the reference (cfr. paragraph 5.3.3), whereas its nasal bioavailability in rabbits was significantly ($P < 0.05$) lower. Thus, further investigations are needed to elucidate the relevance of the elasticity modulus on nasal drug absorption.

Furthermore, in Chapter 4 (cfr. paragraph 4.1.3.1) a similar observation was made: after nasal delivery using the powder (SD 25/75)/$Ca(OH)_2$ 90/10 a higher absorption of insulin was observed in comparison with the SD 25/75 formulation. Besides the higher and faster SNF uptake of (SD 25/75)/$Ca(OH)_2$ 90/10, a higher elasticity but comparable viscosity was obtained after dispersion in SNF in comparison with SD 25/75.

Although water absorption of the SD 25/75 powder during storage at 25 °C - 60 % RH was slightly lower than during storage at 40 °C - 75 % RH (cfr. paragraph 5.3.1) a slower liquid uptake rate was observed (cfr. paragraph 5.3.2). This indicates that storage at elevated relative humidity and temperature is required to transform the polymers to a relaxed state and

to form a super slurper. At higher temperature the flexibility of the hydrated polymer chains will increase and it is suggested that at 40 °C – 75 % RH the polymer chains of Carbopol® 974P have the potential to uncoil whereby their functional groups are preferentially directed towards the surface of the particles. As a consequence the functional groups are immediately available for hydration upon contact with water.

Furthermore, it was observed that after nasal delivery of the reference formulation (batch number 12621: 14F) at t = 0 a bioavailability of only 12.4 ± 2.0 % was observed in comparison with 19.2 ± 5.3 % obtained after nasal delivery of SD 25/75 powder (batch number 10851: 141-1) used in Chapter 3 and 4. To verify the batch variability a third batch of SD 25/75 (batch number 13724: 4A) was prepared, resulting in an even lower bioavailability (7.5 ± 1.8 %) (Table 3).

Table 3. Absolute bioavailability, C_{max} and t_{max} (mean ± SD) after nasal delivery to rabbits of different batches of powder (1IU insulin/mg) containing SD 25/75.

Batch number (SD 25/75)	BA (%)	C_{max} (µIU/ml)	t_{max} (min)	n
10851: 141-1	19.2 ± 5.3	681 ± 247	50.9 ± 7.4	8
12621: 14F	12.4 ± 2.0	441 ± 125 *	36.9 ± 5.6	8
13724: 4A	7.5 ± 1.8 ***	352 ± 120 **	31.2 ± 13.0 **	8

Significantly different from original batch (10851:141-1) (* = 0.05 ≥ P > 0.01; ** = 0.01 ≥ P > 0.001 and *** = P ≤ 0.001)

Future work must certainly elucidate the factors which are causing this variability as this is essential for the further development of these carriers for nasal delivery. Several parameters might contribute to this variability as they could have an influence on the characteristics of the final product:

- quality of raw materials (starch and Carbopol®)
- manipulations prior to spray-drying (temperature at which the Amioca® starch and Carbopol® mixture is stirred after jet cooking, stirrer speed and time)

- spray-drying parameters (e.g. inlet temperature, feed rate, solids concentration, aspirator flow and spray air flow)

5.4 Conclusions

Storage of a spray-dried mixture of Amioca® starch and Carbopol® 974P 25/75 at 40 °C – 75 % RH induced structural modifications of the polymers which had an influence on the bioavailability of insulin after nasal delivery to rabbits.

In order to maintain the powder characteristics obtained immediately after spray-drying the mixture of Amioca® starch and Carbopol® 974P 25/75 should be stored at 25 °C and 10 % RH.

5.5 References

Edman P., Bjork E., Microspheres as a nasal delivery system for peptide drugs. J.Contr. Rel. 21 (1992) 165-172.

Illum L., Farraj N., Fisher A., Gill I., Miglietta M., Benedetti D., Hyaluronic acid ester microspheres as a nasal delivery system for insulin. J. Contr. Rel. 29 (1994) 133-141.

Melon J., Contribution à l' étude de l' activité sécrétoire de la muqueuse nasale. Caractères de perméabilité de l'épithélium, composition chimique du mucus, le mécanisme de sa formation dans les conditions normales et au cours des hypersécrétions paroxystiques. Acta Oto-Rhino-Laryngol. Belg. 22 (1967).

Sørensen A., Animal models for buccal and nasal delivery studies. In: Duchêne D. (Ed.), Buccal and nasal administration as an alternative to parenteral administration. European Symposium, Paris, 10-11 Dec, Editions de Santé (1991) 162-173.

United States Pharmacopeia XXIV and The National Formulary 19, United States Pharmacopeial Convention, 2000.

Chapter 6 Spray-dried Amioca® starch/Carbopol® as carrier for nasal delivery of oxymetazoline HCl

6.1 Introduction

Within the scope of this thesis powders were evaluated as a nasal platform for the systemic delivery of high molecular weight drugs. However, as the nasal route can also be used for local drug therapy, this chapter evaluates the potential of powders containing viscosity-enhancing polymers as local drug delivery systems. It was decided to evaluate the carrier consisting of Amioca® starch and Carbopol® 974P as a potential delivery system for the local treatment of nasal congestion via oxymetazoline hydrochloride.

Nasal congestion is a major symptom in patients suffering from a common cold, rhinitis and sinusitis, causing discomfort to the patient and impairing the quality of life (Guarderas, 1996). To relieve the symptoms an oral or topical sympathomimetic decongestant can be used. The latter has the advantage of a selective site of action and a rapid onset of action (Jackson et al., 1997; Stübner et al., 2001).

Long-lasting use of topical sympathomimetic agents is not recommended as they are associated with rebound swelling of the nasal mucosa, possibly caused by down regulation of the α-adrenergic receptors on the nasal vasculature (Jackson et al., 1997). Healthy volunteers treated daily with an oxymetazoline nasal spray developed rhinitis medicamentosa within 10 days, confirming that only short-term use of these agents is indicated (Graf and Juto, 1994). Nasal administration of a decongestant generally induces a maximal vasoconstriction leading to a nutrition deficiency of the mucosa. As a consequence, when the effect of the decongestant has worn off excessive vasodilatation occurs whereby an additional dose of decongestant is required. In order to reduce the administration frequency and thus to reduce the risk of this rebound phenomenon, a full vasoconstriction must be avoided. A mixture of Amioca® starch and Carbopol® 974P might increase the residence time of the formulation in the nasal cavity, aiming to smoothen and prolong the decongestive effect of oxymetazoline

HCl compared to a common nasal spray. As a result one could reduce the administration frequency and the medication dose using these formulations, reducing habituation and the risk on the rebound phenomenon.
Furthermore, most nasal sprays and drops contain a preservative such as benzalkonium chloride, which is membrane damaging (Berg et al., 1995) and can worsen rebound nasal congestion (Graf et al., 1995). As nasal powder formulations do not require any preservative, an additional advantage is offered when a drug for local treatment is incorporated in a dry carrier.

The following clinical study was performed in collaboration with Dr. Joke Huyghe and Prof. Dr. Claus Bachert (*Department of Oto-Rhino-Laryngology, Faculty of Medicine and Health Sciences, Ghent University).*

6.2 Materials and methods

Spray-dried mixtures of Amioca® starch and Carbopol® 974P (ratios: 25/75 and 85/15, w/w) (SD 25/75 (batch number: 10851: 141-1) and SD 85/15 (batch number: 12206: 141A)) were prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Carbopol® 974P was supplied by Noveon (Cleveland, Ohio, USA). Oxymetazoline HCl was obtained from Sigma (Bornem, Belgium). Nesivine® spray (0.05 % (w/v) oxymetazoline HCl) was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

6.2.1 Preparation of powder formulations

After dispersion of a physical blend of SD 25/75 and oxymetazoline HCl in distilled water, 2.0 M NaOH was added until pH 7.4 was reached. To remove the water the aqueous dispersion was freeze-dried using an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to 228 K within 175 min at 1000 mbar. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar during 13 h, followed by secondary drying at elevated temperature (283 K) and reduced pressure

(0.1 - 0.2 mbar) for 7 h. After freeze-drying the powder was sieved (63 μm) at low relative humidity (20 %) and ambient temperature. The fraction below 63 μm was stored in a desiccator at 4 - 8 °C until use.

In addition a physical blend of SD 85/15 and oxymetazoline HCl was dispersed in distilled water. This dispersion was not neutralised prior to freeze-drying.

Both powder formulations were prepared in order to obtain a final concentration of 0.09 % (w/w) oxymetazoline HCl.

6.2.2 Nasal delivery devices

The Monopowder (Valois, Marly-le-Roi, France) and Pfeiffer system (Pfeiffer, Radolfzell, Germany) were used as nasal delivery devices for the powders (Figure 1).

The Monopowder device is a single-dose delivery system. By actuation of the system, a pressure (compression of air) is generated inside the system, this releases the valve sealing the powder container and sprays the powder into the nasal cavity.

The Pfeiffer system is a bi-dose disposable device having a dual storage chamber for the nasal powder. The powder formulation is deposited into the nasal cavity by inhalation.

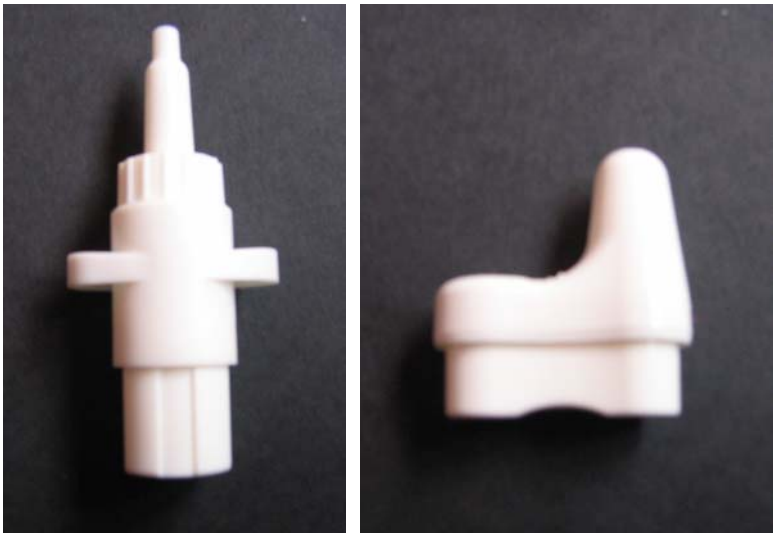

Figure 1. Monopowder device (left) and Pfeiffer system (right).

6.2.3 Quantification of oxymetazoline hydrochloride

Oxymetazoline HCl was quantified using a validated HPLC method with UV-VIS detection. Analysis was performed with a HPLC system consisting of a HPLC pump (LC-10AD-VP, Shimadzu, Antwerp, Belgium), a UV-VIS detector (SPD-10A, Shimadzu, Antwerp, Belgium) and an integrator (C-RSA, Shimadzu, Antwerp, Belgium). The column was a Lichrospher® 60 RP-Select B (125 mm x 4 mm). The mobile phase, used as an isocratic eluent, consisted of 60 % (v/v) 31 mM ammonium sulphate in distilled water and 40 % (v/v) acetonitrile. The eluate was monitored at 220 nm. The retention time of the oxymetazoline HCl peak was 3.5 min at a flow rate of 1.0 ml/min. The analysis was performed at room temperature.
Calibration samples from 2.64 µg/ml to 52.8 µg/ml were prepared in the mobile phase. To 500 mg powder formulation, mobile phase was added until a volume of 10.0 ml was obtained. This dispersion was put in an ultrasonic bath for 5 min and centrifuged (2500g, 10 min). 20 µl supernatant was injected onto the HPLC column.

6.2.4 Study protocol

6.2.4.1 Study design

The study protocol was approved by The Ethics Committee of the Ghent University Hospital. A written informed consent was obtained from each patient.
The study consisted of 3 parts: 2 experiments were designed as a single-blind, cross-over double-treatment test (Exp. I and II), whereas one experiment was a non-blind single-treatment test (Exp. III) in healthy volunteers (n = 6 for Exp. I, n = 4 for Exp. II and n = 5 for Exp. III). Before nasal delivery of Nesivine® and the test formulation the subjects acclimatised to room temperature and relative humidity during 1 h and after administration they stayed during 8 h in the same room. A wash-out period of minimum 2 weeks was respected between successive experiments.

6.2.4.2 Subjects

Four to six healthy volunteers aged between 18 and 50 years, extremes included, participated into the study. The subjects included in the study were healthy as proven by medical history and physical examination.

Patients suffering from chronic diseases such as asthma, diabetes mellitus, hyperthyroid, epilepsy, cardiovascular diseases or liver or renal disorders were excluded from the study. Subjects with an increased intraocular pressure, urinary retention or known alcohol, barbiturate, amphetamine or narcotic abuse were also not selected.

Nasal congestion or obstruction, acute or chronic rhinosinusitis, pharyngeal erythema, fungal infection of the nose, mouth or throat, clinically relevant bronchospasm, primary ciliar dyskinesy or cystic fibrosis were also exclusion criteria.

Volunteers with perennial allergic rhinitis (PAR) or seasonal allergic rhinitis (SAR) could also not participate. Skin prick tests were performed if an allergy was supposed. Individuals having an allergy or hypersensitivity to antihistamines, decongestives or intranasal corticosteroids were also excluded.

Included subjects had no anatomic abnormalities (deviated nasal septum, nasal polyps, atrophic rhinitis or sinusitis, …) of the nasal cavity as determined by anamnesis and clinical examination (rhinoscopy anterior and nasal endoscopy).

With the exception of oral contraceptives, concomitant use of medication during the experiment was not allowed. Administration of an oral, parenteral or nasal short or long acting corticosteroid or nasal cromolyn was prohibited within one month before enrollment in the study, MAO-inhibitors within 2 weeks before and an antihistamine or leukotriene modifier within 5 days before. Subjects with a habitual use of nasal decongestants were also excluded.

Pregnant, lactating females or females with childbearing potential without adequate contraception were excluded from the study. Only non-smokers were included.

During the experiment food containing tyramine (e.g. cheese, wine, herring, …) or tryptophan (e.g. beans) were not allowed.

6.2.4.3 Study medication

6.2.4.3.1 Experiment I

Nesivine® (0.05 % oxymetazoline HCl) was used as reference formulation. One dose (≈ 45 µl or 22.5 µg oxymetazoline HCl/actuation) was sprayed into each nostril. As test formulation a powder containing SD 25/75 (neutralised) and 0.09 % oxymetazoline HCl was used. In order to compensate for the amount of powder remaining into the device after actuation, the

Monopowder device was filled with 25.3 mg of powder. One dose (≈ 25 mg or 22.5 µg oxymetazoline HCl/actuation) was sprayed into each nostril.

6.2.4.3.2 Experiment II

Nesivine® (0.05 % oxymetazoline HCl) was used as reference formulation. One dose (≈ 45 µl or 22.5 µg oxymetazoline HCl/actuation) was sprayed into each nostril. As test formulation a powder containing SD 85/15 (non-neutralised) and 0.09 % oxymetazoline HCl was used. In order to compensate for the amount of powder remaining into the device after actuation, the Monopowder device was filled with 25.3 mg of powder. One dose (≈ 25 mg or 22.5 µg oxymetazoline HCl/actuation) was sprayed into each nostril.

6.2.4.3.3 Experiment III

Nesivine® (0.05 % oxymetazoline HCl) was used as reference formulation. One dose (≈ 45 µl or 22.5 µg oxymetazoline HCl/actuation) was sprayed into each nostril. As test formulation a powder containing SD 85/15 and 0.09 % oxymetazoline HCl was used. In order to compensate for the amount of powder remaining into the device after inhalation, each chamber of the Pfeiffer system was filled with 12.7 mg of powder. Two doses (≈ 12.5 mg or 11.25 µg oxymetazoline HCl/actuation) were inhaled into each nostril.

6.2.5 Efficacy evaluation

6.2.5.1 Nasal airflow

Before rhinomanometry the volunteers needed to avoid major physical activities (e.g. climbing stairs). Rhinomanometry was conducted in sitting position. The subject was asked to blow his/her nose prior to measurement, if necessary. First, a nasal adapter of appropriate size was inserted into the left nostril when measuring the airflow resistance of the right nostril. Subsequently, a mask was pressed on the face for an exact fit, but without distortion of the nostril. The patient had to breathe normally with closed mouth. After a few breaths, data logging was started. After 3 to 5 further breaths the result (nasal volume stream

at 150 Pa) was displayed. The nasal volume stream of the left side was measured accordingly. To ensure correct measurement, baseline adjustment to zero was done without inserted nasal adapter and without putting on the mask. The nasal airflow was recorded 30 min before and 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 8 h after administration of the formulations.

- *Statistical analysis*

The data of nasal airflow are represented as the sum of nasal volume stream of both nostrils (corrected for baseline). The area under the curve (AUC) of the individual nasal airflow versus time profiles was calculated using the trapezoidal method. Statistical analysis of the data was performed using SPSS version 11.0. The data were tested for normal distribution with a Kolmogorov-Smirnov test. To detect statistical differences in nasal airflow between Nesivine® spray and the nasal powder formulation used in Exp. I or II a paired t-test with $P < 0.05$ as significance level was used. To detect a statistical difference in nasal airflow between the nasal powder formulation used in Exp. III and Nesivine® spray administered during Exp. I a one-way Anova was done with $P < 0.05$ as significance level.

6.2.5.2 Nasal symptoms

The severity of nasal symptoms (nasal obstruction, itching and sneezing) was rated 30 min before and 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 8 h after challenge using a visual analogue scale (VAS). Nasal obstruction was assessed on a scale from -10 to 10 (-10 = extremely increased, 0 = normal and 10 = extremely decreased nasal patency). Itching and sneezing were classified on a scale from 0 to 10 (0 = no symptoms and 10 = severe discomfort). For nasal obstruction and itching the right and left nostril were scored individually, but for analysis of the data the mean values were used.

6.2.5.3 Saccharine test

The mucociliary function was evaluated in Exp. II using the indigocarmine saccharin liquid test. After administration of the solution the time interval was determined until the patient perceived a sweet taste or until the physician saw a bluish colour in the oropharynx. The

mucociliary clearance was evaluated 3.5 h after nasal administration of the powder formulation.

- *Statistical analysis*

The computer software SPSS version 11.0 was used for statistical analysis of the data. The data were tested for normal distribution with a Kolmogorov-Smirnov test. A paired t-test with $P < 0.05$ as significance level was used to detect a statistical difference in mucociliary clearance between before and after nasal administration of the powder formulation.

6.2.6 Dialysis experiment

A 10 % (w/w) dispersion of powders containing SD 25/75 or SD 85/15 (cfr. paragraph 6.2.1) was made in simulated nasal fluid (SNF) (7.45 mg/ml NaCl, 1.29 mg/ml KCl and 0.32 mg/ml $CaCl_2$, Melon, 1968). 10 g dispersion was dialysed using a Spectra/Por® 7 membrane with a molecular weight cut off of 2000 Da (Spectrum Laboratories, Breda, The Netherlands). The filled dialysis membrane was submerged in 200 ml SNF, which was stirred using a magnetic stirrer at room temperature. The dispersion was dialysed during 12 h to ensure equilibration of free oxymetazoline HCl between the internal and external medium.
The concentration of oxymetazoline HCl in the extern medium was quantified using the method described in paragraph 6.2.3. Calibration samples in a concentration range between 0.063 and 1.00 µg/ml were prepared in SNF. After dilution of the extern medium, 20 µl was injected onto the HPLC column (n = 2).

6.3 Results and discussion

Prior to start with the in-vivo experiments, the oxymetazoline HCl concentrations in the powders based on SD 25/75 and SD 85/15 and in the Nesivine® spray were quantified at 0.090 ± 0.009 % (w/w), 0.094 ± 0.008 % (w/w) and 0.051 % (w/v), respectively.

6.3.1 Experiment I

In a first experiment the efficacy of the nasal spray (Nesivine®) was compared to the nasal powder containing SD 25/75. The powder was filled into the Monopowder device (Valois). This delivery system was selected based on the results of a nasal deposition and bioavailability study (Callens, 2002). This study compared the Monopowder with the Pfeiffer system: the absolute nasal bioavailability in rabbits after administration of a powder containing Drum Dried Waxy Maize starch and Carbopol® 974P (ratio: 90/10) using the Monopowder system (6.6 ± 2.7 %) was slightly higher than the Pfeiffer system (3.6 ± 1.4 %). By gamma-scintigraphy it was shown that a powder administered with the Pfeiffer device was cleared from the nasal cavity at a faster rate compared to the Monopowder device. These observations combined with a higher depletion of the Monopowder system (1 % of the powder remained in the Monopowder device after actuation compared to 30 % in the Pfeiffer system) were the main reasons for selecting the Monopowder system. Moreover, the operating principle of the Monopowder device is similar to the experimental delivery device used for the in-vivo experiments in rabbits.

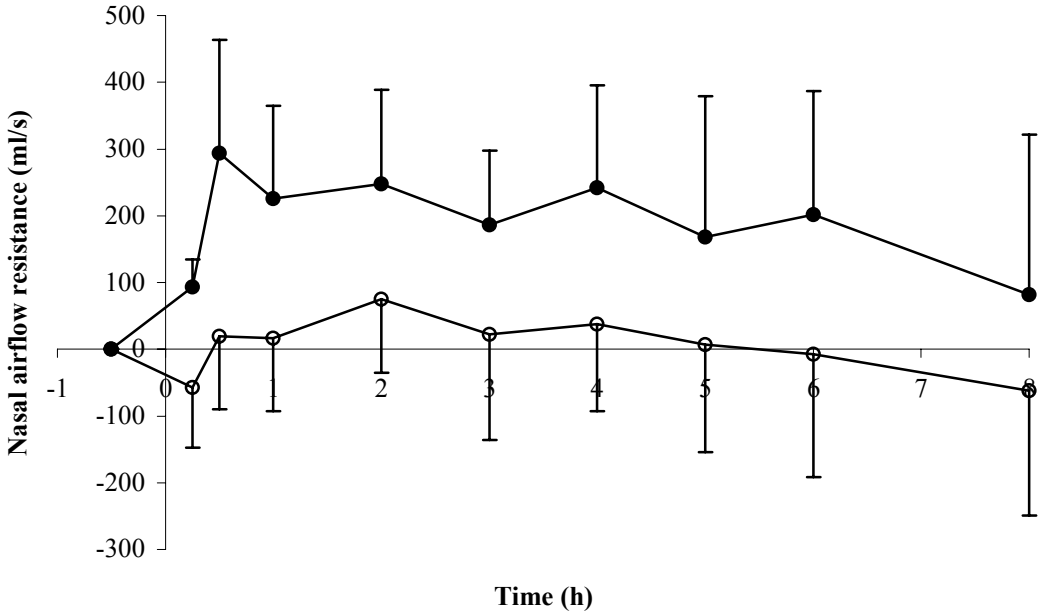

Figure 2. Nasal airflow versus time profile obtained after nasal delivery of a commercial oxymetazoline HCl spray (Nesivine®) (closed symbols) and a powder containing SD 25/75 (neutralised) and oxymetazoline HCl (open symbols) delivered with the Monopowder device to healthy volunteers. Data are presented as mean ± SD (n = 6).

After nasal delivery of the formulations to the volunteers, the evolution of nasal congestion was followed by measuring the nasal airflow through the nostrils using a rhinomanometer. The results are depicted in Figure 2. After nasal delivery of Nesivine® a fast onset of action of oxymetazoline HCl was observed: 15 min after administration a higher nasal airflow was measured, reaching a maximum of 294 ± 170 ml/s after 30 min. The effect was maintained for at least 6 h. In contrast oxymetazoline HCl formulated in the nasal powder based on SD 25/75 induced a lower nasal decongestion compared to Nesivine®. Statistical analysis of the area under the curve demonstrated a significant difference ($P < 0.05$) between both formulations (Table 1). The reduced effect after administration of the powder might be due to an ionic interaction between oxymetazoline HCl and the negatively charged carboxylic acid groups of Carbopol® 974P, preventing drug release from the carrier.

Table 1. Area under curve of nasal airflow versus time profiles obtained after nasal delivery of a commercial oxymetazoline HCl spray (Nesivine®) and of powders containing SD 25/75 (neutralised) or SD 85/15 (non-neutralised) in combination with 0.09 % oxymetazoline HCl.

	AUC (h.ml/s)
Exp. I	
Nesivine® spray	1532 ± 1005
SD 25/75 (Monopowder)	75 ± 1015 [a]
Exp. II	
Nesivine® spray	1524 ± 768
SD 85/15 (Monopowder)	205 ± 663 [a]
Exp. III	
SD 85/15 (Pfeiffer)	1486 ± 477

Data are presented as mean ± SD.

[a] Significantly lower than Nesivine® spray ($P < 0.05$, paired t-test)

Subjective evaluation of the nasal patency using VAS showed that only some volunteers experienced a slight improvement of nasal airflow after administration of Nesivine®

(Table 2). However, it should be stressed that this experiment was performed on healthy volunteers already having a normal nasal airflow. In contrast a decrease of nasal patency was reported by some volunteers after application of the powder formulation.

In Table 3 and 4 the median, minimum and maximum score for the nasal side effects (itching and sneezing) are given for both nasal formulations. It was observed that Nesivine® was well tolerated by all volunteers. On the other hand, treatment with the nasal powder formulation induced itching in most volunteers. This occurred immediately after application of the formulation and was often accompanied by stinging or even watering eyes. Nasal itching lasted until approximately 4 h after administration. Three volunteers reported a slight headache and two of them feeling a pressure at the maxillary sinuses.
As nasal itching occurred immediately after spraying the powder into the nostrils, the pressure under which the powder is released from the device might be too high, whereby the particles are deposited in the lower as well as in the upper parts of the nasal cavity. Scintigraphic research revealed that a bioadhesive powder (DDWM/Carbopol® 90/10) sprayed with the Monopowder device into the nasal cavity of a silicon human nose model, was mainly deposited on the upper wall of the superior turbinate (Callens, 2002). Once powder particles are deposited in the nasal cavity, they absorb water from the nasal mucosa to form a gelatinous network. Swelling of hydrated particles in the narrow cavities of the upper parts of the nose might create a maxillary pressure and induce headache. It has to be elucidated if the side effects reported by the volunteers are related to the formulation itself and/or to the nasal delivery system.

6.3.2 Experiment II

A second experiment was performed to evaluate if an interaction between oxymetazoline HCl and the ionised carboxylic acid groups of the neutralised starch/Carbopol® mixture prevented oxymetazoline HCl to be released from the carrier.

Table 2. The median, minimum and maximum score for nasal obstruction after nasal delivery of the different formulations to healthy volunteers.

	Exp I						Exp II						Exp III		
	Nesivine® spray			SD 25/75 (neutralised) Monopowder device			Nesivine® spray			SD 85/15 (non-neutralised) Monopowder device			SD 85/15 (non-neutralised) Pfeiffer device		
Time (h)	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
-0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.25	0	-3	0.5	0.5	0	1	-2	-2.5	-0.5	0	-2	3.5	0	-2	1.5
0.5	0	-3	0.5	0.5	0	2	-1.75	-3.5	-1.5	0.5	-3	3.5	-1	-2	0.5
1	0	-3.5	0	1	0	2	-1.5	-3.5	-1	0.25	-5	3	0	-2	0
2	0	-3.5	0.5	0	0	1	-0.75	-2	-0.5	0.25	-2	2	0	-2	0
3	0	-3	0	0.5	0	1.5	-0.75	-2.5	0	0	-1	0.5	0	-2	0
4	0	-2	0	0	0	2.5	-0.5	-1	0	0	-1	1.5	0	-2.5	0
5	0	-2.5	0	0	0	2.5	-0.25	-1	0	0.25	-1	1.5	0	-2.5	0
6	0	-2	0	0	0	3.5	-0.25	-1	0	0	-1	0.5	0	-1.5	0
8	0	-2	0	0	0	4.5	-0.5	-1.5	0	0	-1.5	2.5	0	0	0

Table 3. The median, minimum and maximum score for nasal itching after nasal delivery of the different formulations to healthy volunteers.

	Exp I						Exp II						Exp III		
	Nesivine® spray			SD 25/75 (neutralised) Monopowder device			Nesivine® spray			SD 85/15 (non-neutralised) Monopowder device			SD 85/15 (non-neutralised) Pfeiffer device		
Time (h)	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
-0.5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
0.25	0	0	0.5	1.75	0	3.5	1.5	0	5.5	4	4	10	0	0	1
0.5	0	0	0.5	2	0	3	1.25	0	3	2.75	2	10	0	0	1
1	0	0	0	1	0	3.5	0.75	0	1	1.5	1	10	0	0	0
2	0	0	0	0.25	0	1.5	0	0	0.5	0.5	0	8	0	0	0
3	0	0	0	0.25	0	1	0	0	0	0	0	1	0	0	0
4	0	0	0	0.25	0	0.5	0	0	0	0	0	0.5	0	0	0
5	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0

Table 4. The median, minimum and maximum score for nasal sneezing after nasal delivery of the different formulations to healthy volunteers.

	Exp I						Exp II						Exp III		
	Nesivine® spray			SD 25/75 (neutralised) Monopowder device			Nesivine® spray			SD 85/15 (non-neutralised) Monopowder device			SD 85/15 (non-neutralised) Pfeiffer device		
Time (h)	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
-0.5	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
0.25	0	0	1	0.5	0	4	0	0	0	1.5	0	5	0	0	0
0.5	0	0	0	0	0	1	0	0	0	1.5	0	5	0	0	0
1	0	0	0	0	0	4	0	0	0	2	0	3	0	0	0
2	0	0	0	0	0	1	0	0	0	1	0	3	0	0	0
3	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

A non-neutralised SD 85/15 powder has been reported to exhibit bioadhesive capacities when used as a buccal bioadhesive tablet for systemic delivery of testosterone in dogs (Ameye et al., 2005). This mixture was also used as a platform for vaginal controlled release tablets of metronidazole in humans (Ameye, 2004) and for ocular mini tablets having a sustained release of sodium fluorescein in healthy human volunteers (Weyenberg et al., 2005).
As the bioadhesive properties of SD 85/15 have clearly been demonstrated, it was decided to incorporate oxymetazoline HCl in non-neutralised SD 85/15 in order to reduce the risk of ionic interactions between drug and carrier. Furthermore, SD 85/15 is a safe mucosal carrier for drug delivery as it has no irritating or membrane damaging properties (Adriaens et al., 2003).

The nasal airflow versus time profiles of the cross-over study wherein the efficacy of the nasal spray Nesivine® was compared to the nasal powder containing SD 85/15 are shown in Figure 3.

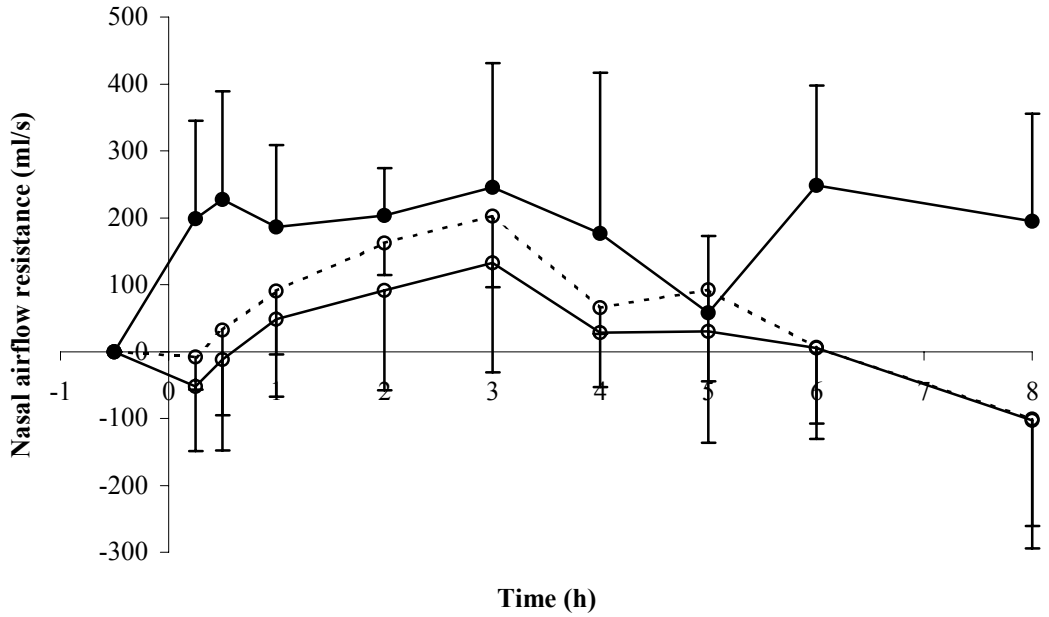

Figure 3. Nasal airflow versus time profile obtained after nasal delivery of a commercial oxymetazoline HCl spray (Nesivine®) (closed symbols) (n = 4) and a powder containing SD 85/15 (non-neutralised) and oxymetazoline HCl (open symbols) delivered with the Monopowder device to healthy volunteers (n = 4 (—) and n = 3 (i.e. the data of the volunteer complaining of pain after nasal administration were omitted) (---)). Data are presented as mean ± SD.

In this experiment a distinct difference between the profile of Nesivine® and the nasal powder formulation could be observed. For Nesivine® a similar profile was seen as in the first experiment: a maximum nasal airflow of 227 ± 162 ml/s was obtained after 30 min, the effect lasting for 6 to 8 h. For the nasal powder formulation SD 85/15 a slower onset of action of oxymetazoline HCl was observed compared to Nesivine®. In case of the powder, the powder must hydrate before the drug can dissolve and become effective. Besides, after hydration of the particles a bioadhesive gel is formed whereby its polymer chains may interpenetrate into the mucus macromolecules, forming a physically entangled network. Also non-covalent bonds between the entangled chains may occur. As a result the diffusion rate of dissolved oxymetazoline HCl molecules through the hydrated Amioca® starch/Carbopol® 974P network could be slower.

These elements may contribute to the delayed onset of action of oxymetazoline HCl after powder administration. For this formulation a maximum nasal airflow of 133 ± 164 ml/s was reached after 3 h. Afterwards the nasal airflow decreased immediately, indicating that the effect could not be maintained using this formulation. This can be explained by the fact that part of the oxymetazoline HCl is cleared from the nasal cavity before the drug molecules can reach their site of action as they remain entrapped in the gel.

Furthermore, the formation of a bioadhesive gel could have prolonged the absorption of oxymetazoline HCl, but assessment of the mucociliary clearance using the saccharine test demonstrated that if there was an initial delay in mucociliary clearance, the normal mucociliary clearance rate had recovered 3.5 h after nasal administration of the SD 85/15 powder formulation as no statistical difference ($P > 0.05$) was found between the mucociliary clearance before administration (20.3 ± 8.2 min) or 3.5 h after administration (18.4 ± 6.6 min).

A significant difference ($P < 0.05$) in AUC-values of the nasal airflow versus time profiles of Nesivine® and SD 85/15 powder was observed (Table 1). However, it should be mentioned that one of the volunteers was complaining of pain after nasal delivery of the powder formulation, causing itching, stinging and watering eyes, pressure at the maxillary sinuses and severe headache. Only in case of this volunteer a decrease in nasal airflow was observed after administration of the powder formulation. Hereby the efficacy reported for SD 85/15

might be underestimated, especially since only 4 volunteers participated in the study (Figure 3).

When comparing the powder formulations containing neutralised SD 25/75 and non-neutralised SD 85/15, a slightly increased nasal airflow was observed for the latter. This might hint at an ionic interaction between oxymetazoline HCl and the carboxylic acid groups of Carbopol® 974P. However, after dialysis of SD 25/75 and SD 85/15 in simulated nasal fluid similar equilibrium concentrations of oxymetazoline HCl in the external medium were obtained: 3.37 ± 0.09 µg/ml and 3.37 ± 0.22 µg/ml, respectively, indicating a release of ± 79 %. So if any interaction occurred between SD 25/75 and oxymetazoline HCl, it is a weak one whereby oxymetazoline HCl is able to dissociate from the ionic complex. However, even a weak interaction may be relevant in nasal in-vivo studies as the formulation may be cleared before the drug was dissociated from the carrier.

6.3.3 Experiment III

To investigate the influence of the delivery system on the nasal patency induced by oxymetazoline HCl formulated in a powder formulation, a third experiment was performed with the disposable system of Pfeiffer. The nasal airflow versus time profile obtained after nasal delivery of SD 85/15 to five healthy volunteers is shown in Figure 4. For comparison the results obtained after administration of the Nesivine® spray (Exp. I) are also shown.

It can be observed that using the Pfeiffer system a similar maximum nasal airflow (227 ± 97 ml/s) was reached compared to the Nesivine® spray. However, the onset of action of oxymetazoline HCl formulated in the SD 85/15 powder was not as steep as in the Nesivine® spray. This can be explained by the fact that oxymetazoline HCl had to diffuse through the hydrated gel formed in the nasal cavity. Furthermore, the effect of oxymetazoline HCl was maintained during 6 to 8 h. The similar efficacy of SD 85/15 after deposition with the Pfeiffer system and the Nesivine® spray was reflected in their AUC of the nasal airflow versus time which were not significantly different ($P > 0.05$) (Table 1).

As already mentioned in literature, the deposition of a formulation in the nasal cavity has an important influence on the efficacy of nasally administered drugs. Also in this experiment it is demonstrated that the delivery system is a critical parameter in the development of nasal formulations as a higher response was obtained with the Pfeiffer device compared to the Monopowder system.

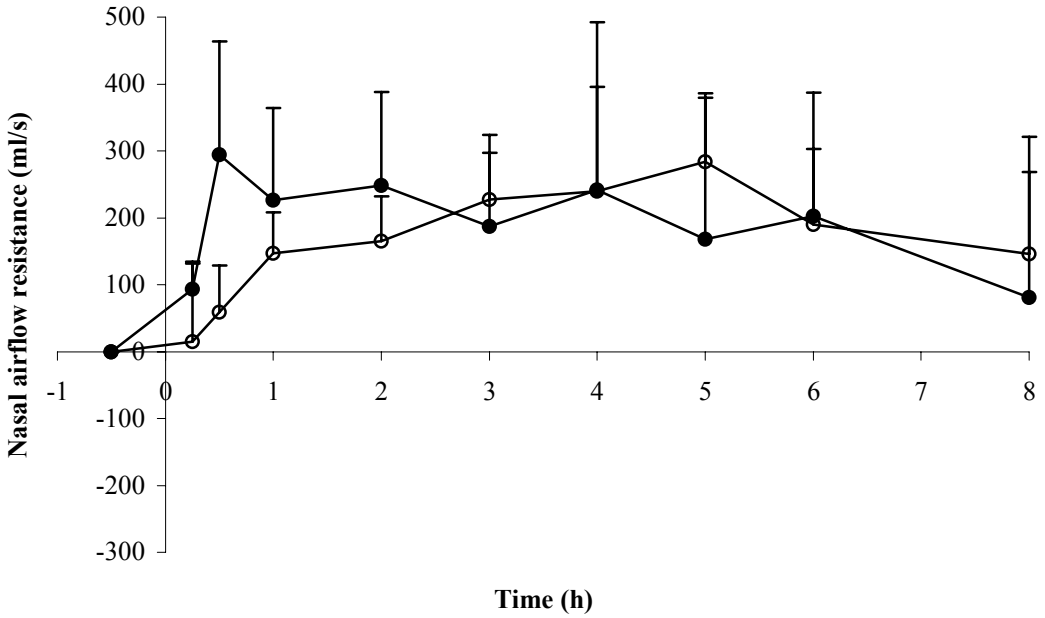

Figure 4. Nasal airflow versus time profile obtained after nasal delivery of a powder formulation based on SD 85/15 (non-neutralised) (open symbols) delivered with the Pfeiffer device to healthy volunteers (n = 5). The nasal airflow versus time profile obtained after nasal delivery of a commercial spray Nesivine® obtained in Exp. I is added for comparison. Data are presented as mean ± SD.

Callens (2002) compared the deposition of a powder (DDWM/Carbopol® 974P 90/10) using the Monopowder and Pfeiffer device in a silicone human nose model and determined that 22 % left the nose model via the pharynx using the Monopowder device compared to only 2 % with the Pfeiffer device. Moreover, 21 % of the powder was deposited at the nasal pharynx with the Monopowder system and only 4.8 % with the Pfeiffer system. The Pfeiffer system distributed the powder homogenously over the turbinates, whereas with the Monopowder device the powder was mainly situated on the upper wall of the superior turbinate. As a result one can assume that less powder will be deposited posteriorly and a

larger fraction will deposite in the absorptive region (inferior and middle turbinates) when using the Pfeiffer device, enhancing and prolonging the decongestive effect.
Only two volunteers reported some nasal itching immediately after administration with the Pfeiffer system, while no sneezing was reported. As the nasal side effects were minimal with the Pfeiffer system it might be concluded that the side effects reported in Exp. I and II are probably related to the nasal delivery system.

The major disadvantage of the Pfeiffer delivery system is the incomplete depletion of the delivery system. As the volunteers were asked to sniff up the entire content of the powder chamber, multiple inhalations were needed to deplete the delivery device.

6.4 Conclusions

In this study it has been demonstrated that the selection of an appropriate delivery system is a critical issue in the development of nasal powder formulations. Incorporation of oxymetazoline HCl in a powder formulation based on non-neutralised SD 85/15 and using the Pfeiffer delivery system resulted in similar pharmacological effect in healthy volunteers as the commercial Nesivine® spray (0.05 % w/v), although the onset of action of oxymetazoline HCl was not as steep when the powder formulation was administered. Longer measurements (> 8 h) are required to verify if a powder formulation based on spray-dried Amioca® starch and Carbopol® 974P offers an advantage (such as longer decongestion, dose reduction) compared to conventional sprays used in the treatment of nasal congestion.

6.5 References

Adriaens E., Ameye D., Dhondt M.M.M., Foreman P., Remon J.P., Evaluation of the mucosal irritation potency of co-spray dried Amioca®/poly(acrylic acid) and Amioca®/Carbopol® 974P mixtures. J. Contr. Rel. 88 (2003) 393-399.

Ameye D., Multifunctional polymers for oral peptide and bioadhesive drug delivery. Doctoral thesis, Ghent University, Belgium, 2004.

Ameye D., Mus D., Foreman P., Remon J.P., Spray-dried Amioca® starch/Carbopol® 974P mixtures as buccal bioadhesive carriers. Int. J. Pharm. 301 (2005) 170-180.

Berg O.H., Henriksen R.N., Steinsväg S.K., The effect of a benzalkonium chloride-containing nasal spray on human respiratory mucosa in vitro as a function of concentration and time of action. Pharmacol. Toxicol. 76 (1995) 245-249.

Callens C., Ontwikkeling van een platform voor nasale toediening van peptiden. Doctoral thesis, Ghent University, Belgium, 2002.

Graf P., Juto J.E., Decongestion effect and rebound swelling of the nasal mucosa during 4-week use of oxymetazoline. Oto-Rhino-Lary. 56 (1994) 157-160.

Graf P., Hallen H., Juto JE., Benzalkonium chloride in a decongestant nasal spray aggravates rhinitis medicamentosa in healthy volunteers. Clin. Exp. Allergy 25 (1995) 395-400.

Guarderas J., Rhinitis and sinusitis: office management. Mayo Clin. Proc. 71 (1996) 882-888.

Jackson R., Todd N., Turner J., Ear, nose and throat diseases. In: Speight T., Holford N. (Eds.), Avery's drug treatment, 4th edition, Adis International, Auchland, 1997, 551-580.

Melon J., Contribution à l' étude de l' activité sécrétoire de la muqueuse nasale. Caractères de perméabilité de l'épithélium, composition chimique du mucus, le mécanisme de sa

formation dans les conditions normales et au cours des hypersécrétions paroxystiques. Acta Oto-Rhino-Laryngol. Belg. 22 (1967).

Stübner U., Toth J., Marks B., Berger U., Burtin B., Horak F., Efficacy and safety of an oral formulation of cetirizine and prolonged-release pseudoephedrine versus xylometazoline nasal spray in nasal congestion. Arzneim.-Forsch./Drug. Res. 51 (2001) 904-910.

Weyenberg W., Bozdag S., Foreman P., Remon J.P., Ludwig A., Characterization and in vivo evaluation of ocular minitablets prepared with different bioadhesive Carbopol-starch components. Eur. J. Pharm. Biopharm. (2005) 1-8.

Summary

As peptides are not suitable for oral administration, they are generally administered parenterally. Due to the disadvantages associated with parenteral delivery alternative routes of administration (buccal, nasal, pulmonal, ocular, transdermal, rectal and vaginal route) have been investigated. In the present work, the nasal route was selected as an alternative route for peptide delivery. Despite the advantages related to nasal administration, the bioavailability remains low. In Chapter 1 the barriers to nasal absorption of peptides are described. The strategies which have been described in literature to circumvent the nasal barriers are also mentioned.

This research project focused on powder formulations as carriers for the nasal delivery of peptides, aiming to improve the nasal bioavailability by enhancing the residence time of the drug in the nasal cavity by incorporating viscosity-enhancing excipients in the formulation. As carrier for the nasal delivery of insulin and salmon calcitonin (used as model peptides) mixtures of Drum Dried Waxy Maize starch and Carbopol® 974P (ratio: 95/5, w/w) (DDWM/C 974P 95/5) and of spray-dried Amioca® starch and Carbopol® 974P (ratio: 25/75 and 85/15, w/w) (SD 25/75 and SD 85/15) were used. In Chapter 2 the characteristics of the components of the nasal powders were described in detail.

In Chapter 3 the influence of powder bulk density on the nasal bioavailability of insulin was investigated in rabbits as in a previous study it was shown that the density of the formulation determined the spray pattern from the device. The powder bulk density of the nasal formulations was modified by changing the solid fraction of the dispersion (prior to freeze-drying) and by changing the freezing rate during the freeze-drying cycle. The bulk density was mainly determined by the solid fraction of the dispersion: the bulk density of the powders increased when the solid fraction of the freeze-dried dispersion was higher. However, no influence of powder bulk density on the nasal bioavailability of insulin in rabbits was seen.

In Chapter 4 it was demonstrated that freeze-drying of aqueous dispersions containing spray-dried Amioca® starch/Carbopol® 974P (ratio: 25/75) and $Ca(OH)_2$ neutralised with NaOH yielded powders containing a mixture of sodium and calcium carboxylate. In Chapter 4.1 the absorption enhancement of those powders was compared with equivalent powders containing only sodium carboxylate.

An optimal balance between sodium and calcium carboxylate was required to maximise the absorption of insulin. The highest absorption of insulin was observed at a ratio of 90/10 between SD 25/75 and $Ca(OH)_2$, obtaining a bioavailability of ± 29 % and 19 % for equivalent formulation with and without $Ca(OH)_2$. This increase in nasal delivery was possibly due to the higher water absorbing capacity of the formulation (enhancing the paracellular transport by opening of the tight junctions) and to the higher elasticity after dispersing this formulation in nasal fluid. Furthermore, after nasal delivery of (SD 25/75)/$Ca(OH)_2$ 90/10 or (SD 25/75)/$CaCO_3$ 90/10 a decrease in t_{max} was observed, possibly due to a progressive dissociation of Ca^{2+}-ions from the Ca^{2+}-carboxylate after hydration of the powder which resulted in the closing of the tight junctions.

Incorporation of salmon calcitonin in the (SD 25/75)/$CaCO_3$ 90/10 formulation resulted in a similar trend: a higher C_{max} but lower t_{max} was observed compared to the equivalent formulation without $CaCO_3$.

The importance of using a spray-dried mixture of starch and Carbopol® 974P instead of a physical mixture was also highlighted in this chapter. A physical mixture of starch and Carbopol® induced a lower absorption of insulin, possibly due to its lower and slower water absorbing capacity. A similar observation was made after addition of $Ca(OH)_2$ to this mixture: C_{max} increased and t_{max} decreased in comparison with the mixture without $Ca(OH)_2$.

As an enhanced bioavailability is frequently associated with a disruption of the integrity of the mucosal epithelium, in Chapter 4.2 it was investigated if the enhanced nasal bioavailability of insulin after administration of the powder formulation containing (SD 25/75)/$CaCO_3$ 90/10 was due to mucosal damage. The possible toxicological effects after multiple administration of the powders SD 25/75 and (SD 25/75)/$CaCO_3$ 90/10 were evaluated using a non-invasive washing technique in rabbits and using a mucosal irritation assay on slugs.

Both powder formulations induced mucosal damage after nasal administration to rabbits as an increased protein, LDH and ALP release from the nasal mucosa after administration was

observed. A higher membrane damaging potential was observed for SD 25/75 compared to (SD 25/75)/$CaCO_3$ 90/10. For both powders the effects on the epithelium were reversible as the protein and enzyme concentrations decreased within 24 h, returning to their basal levels within 3 days.

Using the Slug Mucosal Irritation test, a lower mucosal tolerance was also observed for SD 25/75 as the mucus production and protein and LDH release after repeated administrations were higher in comparison with (SD 25/75)/$CaCO_3$ 90/10. However, according to the Slug Mucosal Irritation prediction model both formulations were classified as moderate membrane irritating and mild to moderate membrane damaging. Hence they are not recommended for chronic drug therapies.

Two modifications of the Slug Mucosal Irritation test were also described in Chapter 4.2, which were performed in order to optimise the procedure for screening of formulations containing viscosity-enhancing and/or bioadhesive polymers. The results obtained using the modified protocols suggested that the present procedure might underestimate the tissue damaging potential of these formulations, especially for those test formulations at the borderline between a mild and moderate classification. The modified procedure wherein the foot mucosa of the slug was carefully cleaned with paper moistened with PBS after the 30-min contact period with the formulation allowed the best prediction of those test substances. Consequently, this procedure will be further optimised and validated.

The stability of the spray-dried Amioca® starch/Carbopol® 974P 25/75 mixture stored during 9 months at different relative humidities (RH) and temperatures (10% RH – 25°C, 60% RH –25°C, 75% RH – 40°C) was investigated in Chapter 5. Storage of a spray-dried mixture of Amioca® starch and Carbopol® 974P 25/75 at 40°C – 75% RH induced structural modifications of the polymers which had an influence on the bioavailability of insulin after nasal delivery to rabbits. In order to maintain the powder characteristics obtained immediately after spray-drying the mixture should be stored at 25°C and 10 % RH.

In Chapter 6 the efficacy of oxymetazoline HCl formulated in a starch/Carbopol® powder formulation was compared to a commercial nasal spray (Nesivine®) in healthy human volunteers by measuring the nasal airflow during 8 h after administration and by scoring the nasal side effects (nasal obstruction, itching and sneezing) using a visual analogue scale. Powder formulations based on neutralised Amioca® starch/Carbopol® 974P 25/75 and non-

neutralised Amioca® starch/Carbopol® 974P 85/15 were used as carriers for oxymetazoline HCl and two systems (Monopowder and Pfeiffer) were used as delivery devices. Nasal delivery of the neutralised SD 25/75 powder using the Monopowder device hardly improved the nasal airflow, possibly due to an ionic interaction between drug and carrier. In contrast, an increase in nasal airflow was seen for the non-neutralised SD 85/15 powder. The delivery device had an important influence on the pharmacological response. Spraying SD 85/15 with the Pfeiffer device resulted in higher pharmacological effect in healthy volunteers, the response being similar to the effect of the Nesivine® spray. However, the onset of action of oxymetazoline HCl was not as steep when the powder was administered. Longer measurements (> 8 h) are required to verify if a powder formulation based on Amioca® starch and Carbopol® 974P induced a longer nasal decongestion compared to the conventional sprays.

Furthermore, the side effects reported following powder administration were possibly related to the nasal delivery system: using the Pfeiffer system the nasal side effects were minimal, whereas with the Monopowder device stinging and watering eyes were reported immediately after administration. Possibly the pressure at which the powder particles are released from the device is too high.

Samenvatting

Aangezien peptiden niet geschikt zijn voor orale toediening, worden ze gewoonlijk parenteraal toegediend. Door de nadelen verbonden aan parenterale toediening werden alternatieve toedieningsroutes (de buccale, nasale, pulmonale, oculaire, transdermale, rectale en vaginale route) onderzocht. In de huidige studie werd de nasale route geselecteerd als alternatieve route voor peptidentoediening. Ondanks de voordelen van de nasale toedieningsweg, blijft de biologische beschikbaarheid laag. In Hoofdstuk 1 worden de barrières voor de nasale absorptie van peptiden besproken. De strategieën die in de literatuur beschreven zijn om deze nasale barrières te omzeilen worden eveneens vermeld.

Dit onderzoeksproject focuste zich op poederformulaties die gebruikt werden als drager voor de nasale toediening van peptiden, met als doel de nasale biologische beschikbaarheid te verbeteren door het verlengen van de verblijftijd van het geneesmiddel in de nasale holte door incorporatie van viscositeitsverhogende excipientia in de formulatie. Als drager voor de nasale toediening van insuline en zalmcalcitonine (gebruikt als modelpeptiden) werden mengsels gebruikt van Drum Dried Waxy Maize zetmeel en Carbopol® 974P (ratio: 95/5, g/g) (DDWM/C 974P 95/5) en van gesproeidroogde Amioca® zetmeel en Carbopol® 974P (ratio: 25/75 en 85/15, g/g) (SD 25/75 en SD 85/15) gebruikt. In Hoofdstuk 2 werden de karakteristieken van de componenten van de nasale poeders in detail beschreven.

In Hoofdstuk 3 werd de invloed van de poeder bulkdensiteit op de nasale biologische beschikbaarheid van insuline onderzocht in konijnen aangezien in een voorgaande studie aangetoond was dat de poederverneveling bepaald werd door de densiteit van de formulatie. De poeder bulkdensiteit van de nasale formulaties werd gewijzigd door het veranderen van de fractie aan vaste stof van de dispersie (alvorens te vriesdrogen) en door het veranderen van de invriessnelheid tijdens de vriesdroogcyclus. De bulkdensiteit werd hoofdzakelijk bepaald door de fractie aan vaste stof van de dispersie: de bulkdensiteit van de poeders nam toe wanneer de fractie aan vaste stof in de te vriesdrogen dispersie hoger was. Hoewel, geen

invloed van de poeder bulkdensiteit op de nasale biologische beschikbaarheid van insuline werd waargenomen in konijnen.

In Hoofdstuk 4 werd aangetoond dat vriesdrogen van waterige dispersies die gesproeidroogd Amioca® zetmeel/Carbopol® 974P (ratio: 25/75) en $Ca(OH)_2$ geneutraliseerd met NaOH bevatten, resulteerden in poeders bestaande uit een mengsel van natrium en calcium carboxylaat. Het absorptieverhogend vermogen van deze poederformulaties werd in Hoofdstuk 4.1 vergeleken met de equivalente poeders die enkel een natrium carboxylaat bevatten.
Een optimale balans tussen natrium en calcium carboxylaat was noodzakelijk om een verhoging in absorptie van insuline te bekomen. De hoogste absorptie van insuline werd waargenomen voor een ratio 90/10 tussen SD 25/75 en $Ca(OH)_2$, waarbij een biologische beschikbaarheid van ± 29 % en 19 % bekomen werd voor equivalente formulaties met en zonder $Ca(OH)_2$. Deze stijging in nasale toediening was mogelijks te wijten aan de hogere waterabsorberende capaciteit van de formulatie (verhoging van het paracellulair transport door het openen van de tight junctions) en aan de hogere elasticiteit na dispersie van deze formulatie in neusvocht. Verder werd na nasale toediening van (SD 25/75)/$Ca(OH)_2$ 90/10 of (SD 25/75)/$CaCO_3$ 90/10 een daling in t_{max} waargenomen, mogelijks te wijten aan een progressieve dissociatie van Ca^{2+}-ionen van het Ca^{2+}-carboxylaat na hydratatie van het poeder resulterend in het sluiten van de tight junctions.
Incorporatie van zalmcalcitonine in de (SD 25/75)/$CaCO_3$ 90/10 formulatie resulteerde in een vergelijkbare trend: een hogere C_{max} maar lagere t_{max} werd waargenomen in vergelijking met de equivalente formulatie zonder $CaCO_3$.
Het belang van het gebruik van een gesproeidroogd mengsel van zetmeel en Carbopol® 974P in plaats van een fysisch mengsel werd ook belicht in dit hoofdstuk. Een fysisch mengsel van zetmeel en Carbopol® induceerde een lagere absorptie van insuline, mogelijks te wijten aan zijn lager en trager waterabsorberend vermogen. Een vergelijkbare observatie werd waargenomen na toevoeging van $Ca(OH)_2$ aan dit mengsel: C_{max} nam toe en t_{max} daalde in vergelijking met het mengsel zonder $Ca(OH)_2$.

Aangezien een verhoogde biologische beschikbaarheid frequent geassocieerd wordt met het verbreken van de integriteit van het mucosale epitheel, werd in Hoofdstuk 4.2 onderzocht of de verhoogde nasale biologische beschikbaarheid van insuline na toediening van de

poederformulatie die (SD 25/75)/$CaCO_3$ 90/10 bevatte te wijten was aan mucosale beschadiging. De mogelijke toxicologisch effecten na multipele toediening van de poeders SD 25/75 en (SD 25/75)/$CaCO_3$ 90/10 werden geëvalueerd gebruik makend van een niet-invasieve spoeltechniek in konijnen en gebruik makend van een mucosale irritatie test op slakken.

Beide poederformulaties induceerden mucosale beschadiging na nasale toediening aan konijnen aangezien een verhoogde vrijgave van proteïnen, LDH en ALP vanuit de nasale mucosa werd waargenomen na toediening. Een hoger membraanbeschadigend vermogen werd waargenomen voor SD 25/75 in vergelijking met (SD 25/75)/$CaCO_3$ 90/10. Voor beide poederformulaties waren de effecten op het epitheel reversiebel aangezien de concentratie aan proteïnen en enzymen daalde binnen de 24 h, terugkerend naar hun basale levels binnen de 3 dagen.

Gebruik makend van de mucosale irritatietest op slakken werd een lagere mucosale tolerantie waargenomen voor SD 25/75 aangezien de mucusproductie en de vrijgave van proteïnen en LDH na herhaalde toediening hoger was in vergelijking met (SD 25/75)/$CaCO_3$ 90/10. Hoewel, volgens het predictiemodel van de mucosale irritatietest op slakken werden beide formulaties geclassificeerd als matig membraanirriterend en mild tot matig membraanbeschadigend. Bijgevolg worden ze niet aanbevolen voor chronische geneesmiddeltherapieën.

Twee modificaties van de mucosale irritatietest op slakken werden ook beschreven in Hoofdstuk 4.2. Deze testen werden uitgevoerd met het oog op het optimaliseren van de procedure van de screening van formulaties die viscositeitsverhogende en/of bioadhesieve polymeren bevatten. De resultaten verkregen met de gewijzigde procedures gaven een indicatie dat de huidige procedure het weefselbeschadigend vermogen van deze formulaties zou onderschatten, vooral voor die testformulaties die zich bevinden op de grens van de milde en matige klasse. De gemodificeerde procedure waarin de voetmucosa van de slak voorzichtig werd schoongemaakt met PBS bevochtigd papier na de contactperiode van 30 min met de formulatie, liet de beste predictie toe van deze testsubstanties. Bijgevolg zal deze procedure verder geoptimaliseerd en gevalideerd worden.

De stabiliteit van het gesproeidroogde Amioca® zetmeel/Carbopol® 974P 25/75 mengsel bewaard gedurende 9 maanden bij verschillende relatieve vochtigheden (RV) en temperaturen (10% RV – 25°C, 60% RV – 25°C, 75% RV – 40°C) werd onderzocht in

Hoofdstuk 5. Bewaring van een gesproeidroogd mengsel van Amioca® zetmeel en Carbopol® 974P 25/75 bij 40°C – 75% RV induceerde structurele wijzigingen van het polymeer die een invloed hadden op de biologische beschikbaarheid van insuline na nasale toediening aan konijnen. Met het oog op het behouden van de karakteristieken van het poeder bekomen onmiddellijk na sproeidrogen moet het mengsel bewaard worden bij 25°C en 10 % RV.

In Hoofdstuk 6 werd de effectiviteit van oxymetazoline HCl geformuleerd in een poederformulatie opgebouwd uit zetmeel/Carbopol® vergeleken met een commerciële nasale spray (Nesivine®) in gezonde vrijwilligers door het meten van de luchtstroom doorheen de neusholte gedurende 8 h na toediening en door het scoren van de nasale bijwerkingen (nasale obstructie, jeuk en niezen) gebruik makend van een visueel analoge schaal. Poederformulaties gebaseerd op geneutraliseerd Amioca® zetmeel/Carbopol® 974P 25/75 en op niet-geneutraliseerd Amioca® zetmeel/Carbopol® 974P 85/15 werden gebruikt als drager voor oxymetazoline HCl en twee systemen (Monopowder en Pfeiffer) werden gebruikt als toedieningsysteem. Nasale toediening van het geneutraliseerde SD 25/75 poeder gebruik makend van het Monopowder toedieningsysteem verhoogde de nasale luchtstroom doorheen de neusholte nauwelijks, mogelijks te wijten aan een ionische interactie tussen het geneesmiddel en de drager. Voor het niet-geneutraliseerde SD 85/15 poeder werd daarentegen een verhoogde nasale luchtstroom waargenomen. Het toedieningsysteem had een belangrijke invloed op de farmacologische respons. Verstuiving van SD 85/15 met het Pfeifer toedieningsysteem resulteerde in een hoger farmacologisch effect in gezonde vrijwilligers, een respons die vergelijkbaar was met het effect van de Nesivine® spray, hoewel de werking van oxymetazoline HCl niet zo snel was wanneer het poeder werd toegediend. Langere metingen (> 8 u) waren nodig om te verifiëren of een poederformulatie gebaseerd op Amioca® zetmeel en Carbopol® 974P een langere nasale congestie induceerden in vergelijking met de conventionele sprays.
Verder waren de gerapporteerde bijwerkingen na toediening van de poederformulaties mogelijks te wijten aan het nasale toedieningsysteem: gebruik makend van het Pfeiffer systeem waren de bijwerkingen minimaal terwijl met het Monopowder systeem onmiddellijk na toediening prikkelende en tranende ogen werden gerapporteerd. De druk waaronder de poederpartikels worden vrijgesteld vanuit het toedieningsysteem was mogelijks te hoog.

Résumé

Puisque les peptides ne peuvent pas être administrés par voie orale, ils sont généralement délivrés par voie parentérale. En raison des désavantages associés à cette administration parentérale, des voies d'administration alternatives (les voies buccale, nasale, pulmonaire, oculaire, transdermique, rectale et vaginale) ont été examinées. Dans cette étude la voie nasale a été sélectionnée comme voie alternative pour l'administration des peptides. Malgré les avantages de cette voie d'administration, la biodisponibilité nasale reste faible. Dans le premier Chapitre les barrières contre l'absorption nasale des peptides sont décrites. Les stratégies décrites dans la littérature pour éviter ces barrières nasales, sont aussi mentionnées.

Ce projet de recherche s'est fixé sur les formulations de poudre qui ont été utilisées pour l'administration nasale des peptides, avec comme but d'améliorer la biodisponibilité en prolongeant le temps de résidence de la formulation dans la cavité nasale par l'incorporation de constituants visqueux. Pour l'administration nasale d'insuline et de calcitonine de saumon (utilisées comme modèles de peptides) des poudres contenant des mélanges de 'Drum Dried Waxy Maize starch' et de Carbopol® 974P (ratio: 95/5, m/m) (DDWM/C 974P 95/5) et des mélanges obtenus par atomisation d'un amidon Amioca® et de Carbopol® 974P (ratio: 25/75 et 85/15, m/m) (SD 25/75 et SD 85/15) ont été employées. Dans le deuxième Chapitre les caractéristiques des constituants des poudres nasales ont été décrites en détail.

Dans le troisième Chapitre l'influence de la densité vrac de la formulation sur la biodisponibilité nasale d'insuline a été examinée chez le lapin puisqu'une étude précédente a montré que la densité vrac de la poudre déterminait le processus de nébulisation de la formulation. La densité vrac des poudres a été modifiée en changeant à la fois la fraction de matière solide de la dispersion (avant lyophilisation) et la vitesse de congélation pendant le cycle de lyophilisation. La densité vrac a été principalement influencée par la fraction de matière solide de la dispersion: la densité vrac des poudres a augmenté quand la fraction de matière solide de la dispersion était élevée. Pourtant, aucune influence de la densité vrac de la poudre sur la biodisponibilité nasale d'insuline n'a été constatée chez le lapin.

Dans le quatrième Chapitre il a été établi que la lyophilisation des dispersions aqueuses contenant un mélange d'un amidon Amioca® et de Carbopol® 974P (atomisé) (ratio: 25/75) et de $Ca(OH)_2$ neutralisé avec NaOH, a conduit à l'obtention de poudres contenant un mélange de carboxylates de sodium et de calcium. La capacité d'augmenter l'absorption d'insuline de ces poudres a été comparée avec des poudres équivalentes ne contenant que du carboxylate de sodium (Chapitre 4.1).

Une balance optimale entre le carboxylate de sodium et de calcium était nécessaire pour obtenir une augmentation de l'absorption d'insuline. L'absorption d'insuline la plus élevée a été observée pour le ratio de 90/10 entre SD 25/75 et $Ca(OH)_2$, permettant d'atteindre une biodisponibilité de ± 29 % et de 19 % pour les formulations équivalentes avec et sans $Ca(OH)_2$. Cette augmentation était probablement due à la plus grande capacité d'absorption d'eau de la formulation (augmentation du transport paracellulaire par l'ouverture des jonctions intercellulaires) et à la plus grande élasticité après la dispersion de cette formulation dans le liquide nasal. Ensuite, après l'administration nasale de (SD 25/75)/$Ca(OH)_2$ 90/10 ou de (SD 25/75)/$CaCO_3$ 90/10 une baisse du t_{max} a été constatée, probablement due à une dissociation progressive d'ions de calcium du carboxylate de calcium après l'hydratation de la formulation résultant dans la fermeture des jonctions intercellulaires.

L'incorporation de calcitonine de saumon dans la formulation (SD 25/75)/$CaCO_3$ 90/10 a conduit à une tendance comparable: C_{max} était augmentée et t_{max} était descendu en comparaison avec la formulation équivalente sans $CaCO_3$.

L'importance de l'usage d'un mélange obtenu par atomisation d'un amidon Amioca® et de Carbopol® 974P au lieu d'un mélange physique a aussi été montrée dans ce chapitre. Un mélange physique d'un amidon Amioca® et de Carbopol® 974P a induit une absorption d'insuline plus faible probablement due à sa capacité plus basse et plus lente d'absorption d'eau. Une observation comparable a été constatée après l'addition de $Ca(OH)_2$ à ce mélange: C_{max} augmentait et t_{max} baissait en comparaison avec la formulation équivalente sans $Ca(OH)_2$.

Vu qu'une biodisponibilité augmentée est fréquemment associée à la rupture de l'intégrité de l'épithélium mucosal, dans le Chapitre 4.2 il a été vérifié si l'augmentation de la biodisponibilité nasale d'insuline après l'administration de la formulation (SD 25/75)/$CaCO_3$ 90/10 était due aux dommages de la muqueuse. Les effets toxicologiques possibles ont été évalués après l'administration multiple des formulations de SD 25/75 et de

(SD 25/75)/$CaCO_3$ 90/10) en utilisant une méthode de rinçage non-invasive chez les lapins et en utilisant un test d'irritation mucosale sur limaces.

Les deux formulations ont induit un dommage mucosal après l'administration nasale aux lapins vu l'augmentation de la sécrétion des protéines, LDH et ALP après l'administration. La formulation SD 25/75 a endommagé davantage les membranes que SD 25/75)/$CaCO_3$ 90/10. Pour les deux poudres les effets sur l'épithélium étaient réversibles, vu que la concentration des protéines et d'enzymes a baissé dans les 24 h, revenant à leur niveau basal en trois jours.

En utilisant le test d'irritation mucosale sur limaces, une tolérance mucosale plus faible a été constatée pour SD 25/75 vu que la production de mucus et la sécrétion des protéines et d'LDH après administration répétée étaient plus élevées en comparaison de (SD 25/75)/$CaCO_3$ 90/10. Pourtant, selon le modèle de prédiction du test d'irritation mucosale sur limaces, les deux formulations ont été classées comme modérément irritantes et endommageant légèrement à modérément les membranes. Par conséquent ces formulations ne sont pas recommandées pour l'administration chronique.

Deux modifications du test d'irritation mucosale sur limaces ont été décrites dans le Chapitre 4.2 qui était effectuées pour optimaliser la procédure de screening des formulations contenant des polymères visqueux et/ou bio-adhésifs. Les résultats obtenus avec les protocoles modifiés ont indiqué que la procédure actuelle sous-estimait le dommage histologique induit par ces formulations, surtout pour celles qui se trouvent dans la classification à la limite entre dommage léger et modéré. La procédure modifiée où la muqueuse des limaces a été nettoyée prudemment avec du papier humecté de PBS après une période de contact de 30 min avec la formulation, a permis la meilleure prédiction du comportement de ces substances. Ainsi la procédure sera optimalisée et validée.

La stabilité du mélange obtenu par atomisation d'un amidon Amioca® et de Carbopol® 974P 25/75 conservé pendant 9 mois à des humidités relatives (RH) et des températures différentes (10% RH – 25°C, 60% RH –25°C, 75% RH – 40°C), a été examinée dans le cinquième Chapitre. La conservation d'Amioca®/Carbopol® 974P 25/75 à 75% RH – 40°C a induit des modifications structurelles des polymères qui avaient une influence sur la biodisponibilité de l'insuline après administration nasale aux lapins. En vue de conserver les caractéristiques de la poudre obtenues immédiatement après atomisation, le mélange doit être conservé à 25°C et 10% RH.

Dans le sixième Chapitre, l'efficacité de l'oxymétazoline HCl formulée dans une poudre composée d' amidon/Carbopol® a été comparée à un nébuliseur commercial (Nesivine®) chez des volontaires en bonne santé en mesurant le courant d'air dans le nez pendant 8 h après l'administration et en quantifiant les effets indésirables (l'obstruction nasale, le prurit et l'éternuement). Oxymétazoline HCl a été incorporée dans des formulations à base d'Amioca®/Carbopol® 974P 25/75 neutralisé et d'Amioca®/Carbopol® 974P 85/15 non-neutralisé et deux systèmes (Monopowder et Pfeiffer) ont été employés comme système d'administration.

L'administration nasale de la poudre SD 25/75 (neutralisé) utilisant le système de Monopowder n'a presque pas augmenté le courant d'air à travers la cavité nasale, probablement à cause d'une interaction ionique entre oxymétazoline HCl et Carbopol® 974P. Par contre, pour la poudre SD 85/15 (non-neutralisé) une augmentation du courant d'air nasal à été constatée. Le système de délivrance avait une influence importante sur l'effet pharmacologique. L'administration de SD 85/15 avec le système de Pfeiffer a induit un effet pharmacologique plus élevé qui était comparable à l'effet du nébuliseur Nesivine®, bien que l'activité de l'oxymétazoline HCl soit moins rapide lors de l'administration de la poudre. Des mesures de plus longue durée (> 8 h) étaient nécessaires pour vérifier si la formulation à base d'amidon Amioca et de Carbopol® 974P provoquait une congestion nasale plus longue par rapport aux nébuliseurs conventionnels.

Ensuite, des effets indésirables rapportés après l'administration des poudres nasales étaient probablement dus au système de délivrance: en utilisant le système de Pfeiffer les effets indésirables étaient minimaux, tandis qu'avec le système de Monopowder, immédiatement après l'administration, on observe un larmoiement et une irritation oculaire. La pression sous laquelle les particules de poudre ont été libérées du système de délivrance est probablement trop élevée.